ISOLATION AND CHARACTERIZATON OF MICROBIAL COMMUNITIES FOUND IN OIL-POLLUTED SITES FROM 1991 GULF WAR

BY

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A Thesis Presented to the

DEANSHIP OF GRADUATE STUDIES

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN, SAUDI ARABIA

In Partial Fulfillment of the
Requirements for the Degree of

MASTER OF SCIENCE

In

ENVIRONMENTAL SCIENCES

MARCH, 2015

KING FAHD UNIVERSITY OF PETROLEUM & MINERALS

DHAHRAN- 31261, SAUDI ARABIA

DEANSHIP OF GRADUATE STUDIES

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DEDICATION

This thesis is dedicated to Allah ﷻ (SWT) and His Rossul, ﷺ محمد

ACKNOWLEDGEMENTS

All thanks and adorations are due to Almighty Allah (SWT) who has always been with me since childhood. He (Allah) alone deserves all appreciations for giving me unquantifiable strength to keep going during this research even when the going becomes very tough. I am also deeply indebted to King Fahd University of Petroleum & Minerals (KFUPM) for providing me scholarship opportunity to sharpen and improve my research skills.

I am very grateful to my advisor, Dr. Assad A. Al-Thukair, the Chairman of Life Sciences Department, for his limitless supports since my arrival on KFUPM up till during the conduct of this research. I appreciate his critical review of my thesis and write-ups. My heartfelt thanks also go to members of my thesis committee, Dr. Alexis M. Nzila (Biology Dept.) and Dr. Basheer Chambasha for their constructive suggestions, material and moral supports.

I must also appreciate the supports of the faculty and staff of Earth Sciences Department starting from the Chairman, Dr. Abdul-Aziz Al-Shaibani, Dr. Bassam S. Tawabini, Prof. Ahmet U. Dogan, Mr. Mushabbab, and others that limited space will not allow me to mention. This research involved numerous analyses conducted in different laboratories with assistance from so many people (and experts). To this end, I must mention Mr. Mohammed H. Omar of Environmental and Chemical Analysis Lab (ECAL) in Center for Environment and Water (CEW), KFUPM Research Institute (R.I), Mr. Monsoor (Chemistry Dept) who trained me in the use of GCMS, Mr. Saravanan (Biology Dept), Mr. AbdulJamiu O. Amao and Mr. Sadaqa (Mech Dept.) for assisting with SEM.

My parents are my congenital sources of diligence and persistence in the face of adversity I cannot thank them enough. They are my sources of joy, just as I am theirs. As my original teachers, their contribution to my life, academic, and research pursuits, is second to none. They showered me with abundant filial love to always keep me spirited at all time. May Allah preserve and bless them (Amin). I also appreciate the incomparable love and supports of my wife (Sariyat) and children, Muhammad Adeshina and Maryam Adejoke who endure only very little time I give to them. I acknowledge the perseverance of my siblings as well.

I cannot find the most appropriate adjectives to eulogize Nigerian Community in KFUPM (NCUPM) for their regular moral and spiritual supports ever before my arrival in Saudi Arabia. It is especially noteworthy that I specifically appreciate Dr. Balarabe Yushau (Mathematical Science Dept) for his role in ensuring I stay to complete my MS program, Dr. Babalola (Earth Sciences/CPM, RI) and other NCUPM leadership. Nearly all NCUPM members contribute one thing or the other to making my stay enjoyable and a success. I appreciate them all.

If I want to dedicate this thesis to mortal, Engr. Sulaiman Lanre Taiwo deserves it. He is more than a brother ever since my first days in OAU. He mentors and supports me in every way. I pray Allah reward him abundantly and bless his family.

This acknowledgement will be incomplete if I fail to mention my friend like a brother, Akeem O. Bello. He is a must-have great companion. Lastly, I am grateful to all my friends for their love, understanding and encouragements to making my study and stay worthwhile. I could have mentioned names but to avoid turning this acknowledgement to a compendium of names. May Almighty Allah bless everyone (Amin). ALHAMDULILLAH!

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LIST OF ABBREVIATIONS

ANOVA	Analysis of Variance
BH	Bushnell Haas media
BLAST	Basic Local Alignment Search Tool
cfu	colony-forming unit
DGGE	Denaturing Gradient Gel Electrophoresis
DNA	Deoxyribonucleic acid
EPAH	Extractable polycyclic aromatic hydrocarbons
G+C	Guanine-cytosine content
GCMS	Gas chromatography fitted with mass spectroscopy detector
HMW	High molecular weight
HPLC	High performance liquid chromatography
LMW	Low molecular weight
NA	Nutrient agar
NCBI	National Center for Biotechnology Information
OD	Optical density
ODM	Oil-degrading microorganism

PAH	Polycyclic aromatic hydrocarbon
PCR	Polymerase chain reaction
PDMS	polydimethyl siloxane
RNA	Ribonucleic acid
rRNA	ribosomal Ribonucleic acid
SEM	Scanning Electron Microscope
SPME	Solid-phase micro extraction
TPH	Total Petroleum Hydrocarbon
USEPA	United States Environmental Protection Agency
UV	Ultraviolet radiation

ABSTRACT

Full Name : [OYEHAN Tajudeen Adeyinka

Thesis Title : [Isolation and Characterization of Microbial Communities found in oil-polluted sites from 1991 Gulf War]

Major Field : [Environmental Sciences]

Date of Degree : [March, 2015]

Arabian Gulf has always suffered a great deal of hydrocarbon pollution, but none was as massive as oil spill resulting from 1991 Gulf War. Remediation of hydrocarbon-contaminated sites is a serious environmental concern due to devastating effects of oil pollution on flora, fauna and general ecosystem. Polycyclic aromatic hydrocarbons (PAHs) deserve special attention because apart from being more toxic, their recalcitrance, hydrophobicity and low volatility make them difficult to degrade. Bacterial degradation has been implicated has a viable and eco-friendly remediation approach. This work therefore aims at isolating and characterizing PAH-degrading bacteria from polluted sites from 1991 Gulf War, and testing their degradation abilities. Using enrichment culture approach, 15 bacterial strains were isolated from sediment samples collected from the contaminated sites. The isolates were characterized morphologically, molecularly and genetically using microscopy, 16S rRNA sequencing and phylogenetic analysis respectively. Ability of selected strains to degrade phenanthrene, pyrene, and diesel components were assessed in liquid cultures analyzed with GCMS after extraction with solid-phase micro-extraction (SPME). The results show that all the isolates were gram-negative rod bacteria belonging to phylum Proteobacteria, with genus *Pseudomonas*

being dominant. Neighbor-joining phylogenetic trees revealed that 87% of the isolates were similar to *P. aeruginosa* while others are relatives (or strains) of *Cupriavidus taiwanensis, P. citronellolis* and *Ochrobactrum intermedium*. Our degradation results showed that isolates BC5 (*P. aeruginosa*) and LB (*P. citronellolis*) were excellent phenanthrene degraders reducing 100 ppm by about 95% within fifteen days, with latter was preferred because of former latent pathogenicity. Isolates BC1 (*O. intermedium*) and DSA (*P. aeruginosa*) were also efficient in degradation of pyrene and diesel components respectively. The isolate DSA could not however degrade aromatic components of diesel significantly. Its increasing order of ease of diesel components' degradation was; *Aromatic* < $(C_8–C_{11})$ < $(C_{12} – C_{14})$ < $(C_{15} – C_{17})$ < $(C_{18} – C_{21})$. The results imply that more than a single strain is needed for effective degradation of diesel. This study also reveals that bacterial growth assessments in PAH-enriched media should not be based on turbidity or optical density of the liquid but on counting of colony-forming units. Conclusively, we report that the bacterial communities in polluted Arabian Gulf coast are diverse and rich in oil degrading strains that can, and should be explored for bioremediation.

ملخص الرسالة

الاسم الكامل: تاج الدين أدينكا وييهان

موضوع البحث: العزل والتوصيف للمجتمعات الميكروبية الموجودة فى المواقع الملوثة بالنفط من حرب الخليج عام ١٩٩١

التخصص : العلوم البيئية

تأريخ التخرج: جمادى الثانى ١٤٣٦

مازال الخليج العربى دائما يعانى قدرا كبيرا من التلوّث الهيدروكاربون، ولكن مابأكثر من التسرب النفطى من هذه التلوثات الناتج من حرب الخليج عام ١٩٩١. ومعالجة المواقع الملوّثة بالهيدروكاربون أمرٌ بيئي مهمٌ نظرا إلى الآثار المدمِّرة المنتجة من التلوّث النفطى على النباتات والحيوانات والنظام البيئى العام. وتستحق الهيدروكاربونات العطرية المتعددة الحلقات (PAH) اهتماما خاصا، لأنه بالإضافة إلى كثرة سُميّتها، فإنّها أصعب تحللا بنشوزها وللامائيتها وانخفاض تقلّباتها. ولقد تورّط تدهور البكتيريا كنهج معالجة مجيدة وصديقة للبيئة. لذا يهد ف هذا البحث إلى توصيف (PAH) المحللة للبكتيريا وعزلها عن المواقع الملوّثة من حرب الخليج ١٩٩١. تمّ عزل ١٥ سلالات من عينات الرواسب التى تمّ جمعها من المواقع الملوّثة. وتميّزت العزلات شكليّا، جزئيّا وراثيّا باستخدام المجهر وتحليل (16S rRNA) الرنا الريباسى التسلسلى والنشوء والتطور على التوالى، ولقد تم تقييم قدرة السلالات المختارة لتحليل فينانترين، بيرين ومكوانات الديزل فى الثقافات السائلة مع (جى سي أم أس) بعد استخراجها مع (أس بي أم إي). وأظهرت النتائج أن جميع العزلات قضيب البكتيريا الغرام السلبية التى تنتمى إلى أسرة بعائلة متقلبات مع كون سيودونومس مادة قاهرة . كشفت أشجار النشوء والتطور-انضمام الجيران أن 87 % من العزلات كانت مشابهة ل(ب. أيروجينوسا) حيث أن الآخرين متقاربة (أو سلالات) من كوبرئفدوس تيوننسس، بي سيطرونيلوليس و أوكروبطروم إنتمي ديعوم. وأظهرت نتائجنا التدهورية أن عزلات بي سي 5 و ل بي سيطرونيلوليس معزلات فينانترين ممتازة تقلص 100 بى بى م بنحو 95 % خلال خمسة عشر يوما مع أفضلية الأخير بسبب مرضية الكامنة السابق. عزلات بي سي 1 (إنتمي ديعوم) و دي أيس أ (ب. أيروجينوسا) أيضا فعالة فى تدهور مكونات بيرين والديزل بالتوالى. ولكن لايمكن ان يتحلل الأخير (عزل دي أيس أ) المكونات العطرية من

مكونات الديزل بشكل جيّد. وكان ترتيب زيادة سهولة مكونات الديزل كالتالى: العطرية $Aromatic < (C_8 - C_{11}) < (C_{12} - C_{14}) < (C_{15} - C_{17}) < (C_{18} - C_{21})$. وتعنى النتائج أن هناك حاجة إلى أكثر من سلالة واحدة لتدهور فعالية من وقود الديزل. تكشف هذه الدراسة أيضا أن تقييمات نمو البكتيريا فى وسائل التخصيب بي أي أيش، لاينبغى أن يكون مبنية على التعكر او الكثافة الضوئية من السائل بل على العد من وحدات تشكيل مستعمرة. اختتاما، تبلغ ان المجتمعات البكتيرية فى ساحل الخليج العربى الملوث منتوّعة وغنية فى سلالات مهينة للنفط وينبغى استكشافها عن المعالجة البيولوجية.

CHAPTER 1

INTRODUCTION

1.1 Background/general introduction

Arabian Gulf has always suffered a great deal of hydrocarbon and other chemical pollution. Being a home for Strait of Hormuz, it is the major route for the transportation of over 60% of global oil production (EIA, 2012), and as such accidental spills from ships, pipelines and tankers are commonplace. However, oil spill from 1991 Gulf War remains the worst environmental pollution with the most devastating marks on the Gulf's ecosystem. About 8 million barrel (252 million US gallons) of crude oil was released into the Sea during the war (Harayama et al., 2004) leading to groundwater and soil contamination (Al-Thukair et al., 2007) as well as air pollution from burnt oil wells (Sadiq and Mian, 1994). The oil spill affects the marine organisms in the Gulf and, the flora and fauna of its coastal areas.

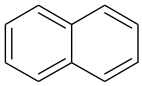
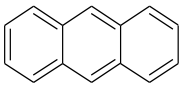
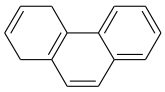
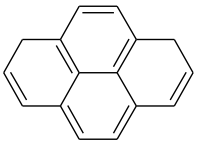
To protect the environment, remediation of oil spill sites is a serious environmental concern to oil-producing nations, oil industries and environmentalists. Different methods of decontaminating environment of hydrocarbons have been developed. However, while some are inefficient, others are either costly (Plata et al., 2008) or completely counter-productive (Yang et al., 2006). Bioremediation is an efficient, cheap and environmentally-safe alternative method which utilizes living organisms to degrade hydrocarbons into a

less toxic or harmless substance(s). This technology is, nevertheless, not fully developed, and is being intensely researched. Most of the available bioremediation technologies can degrade aliphatic components of crude oil efficiently (Whyte et al., 1997), but only little success has been recorded in the removal of polycyclic aromatic hydrocarbons (PAHs) (Kanaly and Harayama, 2000) which are actually the most toxic and likely carcinogenic.

Polycyclic or polynuclear aromatic hydrocarbons (PAHs) are a class of hydrocarbons with two or more fused benzene rings. The aromatic ring(s) in PAHs may contain from four to seven carbon atoms but those having 6-carbon benzene-like (benzenoid) rings are more abundant and environmentally important. The simplest PAH is naphthalene with only two fused benzene rings (Table *1-1*). Although some researchers considers naphthalene as di-cyclic aromatic hydrocarbons and not a PAH (Feng et al., 2009), but numerous chemists consider it the simplest member of PAH (Ferradji et al., 2014; Nair et al., 2008). Generally, PAHs have high boiling and melting points, are hydrophobic, lipophilic, readily soluble in organic solvents (such as n-Hexane, acetone, dichloromethane, etc.) and have unique UV spectra specific for each component ring. Pyrene and higher PAHs are suspected to be carcinogen and, as a result, are of environmental and health concerns.

Oil-degrading microorganisms do not readily mineralize or utilize PAHs because of lack of open terminal alkyl end and insolubility of PAH in water. To achieve appreciable remediation of PAH-contaminated sites, the inoculum (microorganisms) or the medium (environment) should be modified. One way of achieving such modification is isolation of ODM strains from oil contaminated sites (Head et al., 2006).

Table 1-1. Structure and properties of some LMW PAH

Name	*Naphthalene*	*Anthracene*	*Phenanthrene*	*Pyrene*
Formula	$C_{10}H_8$	$C_{14}H_{10}$	$C_{14}H_{10}$	$C_{16}H_{10}$
Structure				
Molecular weight	128.2	178.2	178.2	201.1
Solubility at 25^oC (µg/L)	12500 – 34000	59	435	133
Density (g/cm^3)	1.14	1.18	1.25	1.27
Melting point (^oC)	80.26	101	215.76	145.148
Boiling point (^oC)	218	340	340	404
CAS No.	91-20-3	120-12-7	85-01-8	129-00-0

Bioremediation occurring in nature involves activities of numerous related and unrelated microorganisms interacting in a community. Natural biodegradation, if undisturbed, is however too slow and inefficient to achieve reasonable remediation within short time. Even though the indigenous microbial community of a contaminated site usually consists of nearly all members needed to completely degrade the pollutant, their biological interaction may sometimes be antagonistic and thereby limits remediation success.

Notable oil-degrading bacteria that are commonly isolated from petroleum-contaminated environments are include those in the phyla *Proteobacteria* and *Bacillus* (Deng et al., 2014; Mao et al., 2012; Obayori et al., 2009; Sánchez et al., 2005). *Alcanivorax* and *Cycloclasticus* are two bacterial genera that have been proven to degrade alkanes (straight and branched chains) and aromatic hydrocarbons respectively (Harayama et al., 2004; Head et al., 2006; Teira et al., 2007). *Pseudomonas aeruginosa*, the commonest bacteria in most environment has track record of degradation for both straight chain and aromatic hydrocarbon (Abed et al., 2014; Ghosh et al., 2014; Haritash and Kaushik, 2009; Madueño et al., 2011; Niepceron et al., 2010; Obayori et al., 2009). Some specific strains of these bacteria have been isolated and developed commercially for certain contaminants and toxic hydrocarbons (Andreolli et al., 2011; Chaudhary et al., 2011).

In general, bacterial strains isolated from petroleum-contaminated sites usually possess hydrocarbon-degrading ability. This is because, their successful survival in such a very harsh hydrophobic environments is translated to mean having unique feature of obtaining carbon from the hydrocarbon as source of their energy. Therefore, the first step in bioremediation technology development is strain(s) isolation from polluted sites.

Polluted coastal areas of Arabian Gulf provide suitable sites for such useful microbial communities.

1.2 Research Significance

Increasing energy need and its attendant growing oil production corresponds to considerable pollution of the environment with oil and related chemicals. While there are many methods of removing these harmful substances from the environment, bioremediation remains the cheapest and safest technique. Implementation of this technology requires selection of suitable bacteria that is not only efficient in degradation but also adaptable to the local environment. This research is therefore very significant to pollution management through isolation and characterization of indigenous bio-degrading strains that are adaptable to desert condition of Gulf States. Molecular analysis and characterization of the microbes would foster cloning and laboratory development of the useful indigenous bacteria that can degrade only target hydrocarbon without affecting non-target ones. Finally, this study will contribute to, hitherto unavailable information on likely strains that can be commercialized locally for hydrocarbon remediation.

1.3 Research Objectives

The objectives of this study were;

1. To isolate different strains of bacteria found to degrade polycyclic aromatic hydrocarbons (PAHs).
2. To characterize the isolated strains using molecular techniques and possibly group them phylogenetically.
3. To test the efficiency of the isolated strains in the degradation of specific PAHs.

CHAPTER 2

LITERATURE REVIEW

Since the end of 1991 Gulf War, a number of research has been carried out to assess the magnitude of related oil spill and its effects on the marine and coastal environments, and to design efficient, cost-effective remediation technologies to clean up the affected contaminated sites. In such remediation efforts, polycyclic aromatic hydrocarbons (PAHs) deserve special attention not just because of their toxicity and persistence, but also because of their hydrophobicity and recalcitrance. There is therefore a need to review previous studies on PAHs and their removal, focusing more on how and which microbes have been used to achieve the degradation. The edge of the bioremediation over other remediation technologies would be highlighted and laboratory methods involved in testing and genetically identifying viable bacterial strains would be reviewed with a view to exposing research gap that this study attempt to fill up.

2.1 Oil spill in the Gulf and its environmental effects

The 1991 Gulf war, contributes, so far, the largest source of oil spillage. About 1 million tons of oil was reportedly spilled into the Gulf (Jones et al., 2008; Price and Robinson, 1993; Price et al., 1994). After the spill, the volatile components of the crude rapidly evaporated due to prevailing high temperature and wind in the Gulf. Denser portions form tar balls and settled into the sea bottom and coastline where some of them are still present

till today. Gulf currents, high tides and strong northern winds also move the oil far south and west, taking the contaminating crude to shoreline and far into inland areas (Barth, 2002). Abu Ali Island and Jubail (this research study area) collect a substantial amount of drifting spilled oil reducing the volume that spread further south due to their geography (Figure 2-1). In an assessment of waters, sediment and soil samples from polluted sites within the Gulf by Fowler (Fowler, 1993), following the widespread effects of the spillage, about 260 microgram of petroleum hydrocarbon per liter was found in subsurface seawater, while as high as 18,000 µg/l was recorded in sediment from certain locations.

Figure 2-1. Landsat thematic map showing greasy crude oil (in red) being barricaded by Abu Ali Island and collected within Jubail coastline (Barth, 2002).

Gulf oil spills impact the air (Sadiq and Mian, 1994), the groundwater (Mukhopadhyay et al., 2008), land and marine environments (Price and Robinson, 1993). Many reports indicated that marine ecosystem was most affected. International Union for Conservation

of Nature and Natural Resources (IUCN) (Price et al., 1994) documented the adverse effects of the spill on marine and coastal flora and fauna including fishes, sea urchins, coastal birds, algae, and coral reefs. In contrast to scientists' prediction that spill of Gulf war magnitude in such a shallow water bodies would render the location lifeless, not all biota were significantly affected. For instance, while bird and shrimps populations were reduced substantially, coral reefs were not so much impacted. Even those affected were not below threshold of no recovery. The report did not assess microorganisms.

Loss to microorganisms was initially reported in terms of algal mat in a preliminary pollution assessment report by (Al-Thukair and Al-Hinai, 1993). Even though the mats were seriously affected, they show quick recovery after two years of the spill. Damage to cyanobacteria mat was similarly assessed to be significant (Abed et al., 2006; Al-Thukair et al., 2007). During low tides, leathery mats dry up and crack exposing the tar and oil-rich sediments beneath to intense heat and wind, thereby forming the major remediation process still on-going in the polluted sites (Barth, 2003).

The same cyanobacterial mats also limit microbial remediation to low-efficiency anaerobic process by forming very thick layer over the soil. This constitutes one of the indirect consequences of the spill on other microorganisms. Direct effects on bacteria, fungi and other microbes are erosion of their habitat and suffocation of the aerobic species. Both processes may select only few anaerobic microorganisms and those that can survive toxicity of the hydrocarbon. Nevertheless, when Abed et al. studied microbial diversity of Arabian Gulf mats, a good number of aerobic halo-tolerant and thermo-tolerant hydrocarbon-degrading microbial species were identified (Abed et al., 2006). Prior to their

studies, a review conducted by (Barth, 2002) showed that Arabian Gulf is very rich in hydrocarbon-utilizing microorganisms and therefore has very high potential for bioremediation if limitation posed by low nutrients (mainly nitrogen) can be taken care of.

As pointed out earlier above, heavy portion of the spilled crude oil were removed by deposition into subsurface as tar-balls while volatile components were volatilized and evaporated gradually in the presence of high temperature and strong wind. However, non-volatile hydrophobic components, polycyclic aromatic hydrocarbons (PAHs), cannot be removed by such climatic factors. They remain persistent in sediment, insoluble in water, accumulate in organisms and remain in food chain, and can only be removed efficiently by specially designed remediation technologies. This information is significant considering the fact Kuwait crude oil contains over 16% aromatic compounds or cycloalkanes, of which about 62% are PAHs with 2 – 5 rings (Potter, 1999).

2.2 PAHs as recalcitrant environmental pollutants and their health effects

Polynuclear aromatic hydrocarbon (PAHs) are group of widespread organic pollutants with fused conjugated rings that have no substituents. They are released into the environment mainly through oil spills and incomplete combustion of fossil fuels and organic matter. United States Environmental Protection Agency (USEPA) grouped 16 of them as priority pollutants and classified pyrene, phenanthrene, anthracene and few others as individual priority chemicals (USEPA, 2012) because of their behavior in environment, and their effects on human and animal health.

As non-polar environmental pollutants, they neither volatilize nor mineralize due to their high boiling points and hydrophobicity (Amezcua-Allieri et al., 2012; Gan et al., 2009; USEPA, 2008). The fused rings having no constituents further account for PAHs' resistance to degradation as there is no terminal surface for enzymatic activation in case of biodegradation (McGenity et al., 2012) and for polar reaction. Despite the general belief about PAHs recalcitrance, Boyd et.al (Boyd et al., 2008) observed active aerobic biodegradation of PAH in all the seven sites (modified to about 21 sites). The paper examines influence of closed aromatic ring structure of PAH on its refractory and recalcitrance. It concluded that, initial concentrations rather than aromatic structures are the controlling factors for bioavailability of 2 to 5 rings PAHs. When the concentration is high enough, microbes are stimulated to switch to utilizing PAHs in place of their original carbon source(s). However, since microbes need more than just carbon source to survive, natural PAH bioremediation is not efficient due to lack of nutrients. And therefore, PAHs remain in the environment as serious environmental pollutants and, a refractory carbon and energy sources for microorganisms if that can be harnessed for remediation.

The health effects of PAHs come mainly under the carcinogenity of some of its members. Toxicity of PAHs in lower animals (such as mice) has been confirmed in a study wherein 300 ppm of benzo(a)pyrene (BaP) caused some birth deformations (and liver diseases when the dose is tripled) in mice (USEPA, 2008). The health effects of PAHs are not limited to inducement of cancer, they have also been found to be hepato-toxicants in fish and other mammals (Myers et al., 2003). Although, no studies was found to show

that PAH cause death, cardiovascular and renal diseases, they are suspected as indirect contributors to them.

There is also concern about its mutagenicity and likelihood of causing birth defects. PAHs readily dissolve in lipids and other non-polar solvents. So, on entering the body of organisms, they can penetrate deep through cell membrane eventually getting to chromosomes in the nucleus. While PAHs are not actually mutagens or carcinogens, their metabolites have been linked to mutagenesis and carcinogenesis. A number of studies identified the mutagenic PAH metabolite group as nitro-PAH (Pedersen et al., 2004), that is PAH having nitrogen or nitrogen derivative as substituents. Although, nitro-PAHs are primarily formed from incomplete combustion of PAHs, some are exclusively formed in atmosphere and are everywhere in troposphere (Ramdahl et al., 1986).

Going by the seriousness of health and environmental consequences associated with persistence, hydrophobicity and ubiquity of these pollutants, they must be removed. Their remediation or removal will not only protect our ecosystem but also preserve human health and survival (Haritash and Kaushik, 2009).

2.3 Treatment and removal of PAHs

Having established the severity of PAHs as health hazardous and widespread environmental pollutants, we need to keep their concentrations below contaminant levels. As a matter of fact, environmental agencies such as USEPA, WHO, Saudi Presidency for Metrology and Environment (PME), etc. do not set out a specified

maximum contaminant levels. Oil spill and chemical waste contaminated sites are treated for aromatic compound. Many treatment techniques have been developed and tried (Table *2-1*). Some methods such as chemical oxidation and use of surfactants achieve good results but are very expensive, while others (e.g. photo-oxidation, adsorption, etc.) simply transform the toxic PAHs to other more toxic forms. Bioremediation has the potentials to treat hydrocarbon-polluted sites safely, cheaply and efficiently.

Table 2-1. Some treatment and remediation methods, their main features, advantages and disadvantages

Method	Salient points	Major advantage	Major drawback	References
Volatilization	Requires very high temperature to volatilize	None	Poor and inefficient	(Park et al., 1990)
Photo-oxidation	Use light and oxidants	Efficient in transforming to other species	• Transformed species may be more toxic. • Poor efficiency in *in situ* • Not useful in spill site	(Kou et al., 2007; Plata et al., 2008; Sabaté et al., 2001)
Chemical oxidation	Use oxidants such Fenton's reagent, ozone and/or supercritical water	• Efficient for HMW PAHs • Quick in action	• Expensive • May attack non-target species • May worsen toxicity	(Ferrarese et al., 2008; Rivas, 2006)
Surfactant	Emulsification for bioavailability	Excellent when combine with chemical or microbial methods	Expensive	(Gryzenia et al., 2009; Mulligan et al., 2001)
Adsorption	Utilize surface characteristics to remove PAH	Excellent removal within short duration	• Alter environmental fate • Enhance toxicity	(Gong et al., 2007; Yang et al., 2006)
Phyto-remediation	Plant and its root zone partake in remediation.	Environmentally safe and cost effective	• Climatic adaptation of plant is a concern • Slow	(Meng et al., 2010)
Bio-remediation	Microorganisms utilize PAH as carbon and energy source	• Environment-friendly • Cost effective • Can be *in situ* or *ex situ*	• Slow in nature • May require adaptable local strains	(Abed et al., 2011; Al-Thukair et al., 2007; Nzila, 2013; Wu et al., 2013)

2.4 Biodegradation and Bioremediation: Some previous works

Biodegradation is defined simply as "the natural process whereby bacteria or other microorganisms alter and break down organic molecules into other substances, such as fatty acids and carbon dioxide". While bioremediation is refers to "the act of adding materials to contaminated environments, such as oil spill sites, to accelerate natural biodegradation process" (U.S. Congress, 1991). In other words, biodegradation occurs in nature without intervention while bioremediation is the technology that utilizes the inbuilt biodegradation abilities of microbes to clean polluted environments.

Bioremediation is most favored remediation technique for oil spill and persistent PAH. Recently, attention has been focused on establishing and improving it through attempts to deeply understand the microbiology and ecology of the degraders. After oil spill, all biotas will be affected. Microorganisms are usually the first colonizers.

2.4.1 Succession and community structure

Scientists have previously studied microbial succession in oil pollution sites. Kaplan and Kitts (Kaplan and Kitts, 2004) monitored bacteria succession and community structure during total petroleum hydrocarbon (TPH) degradation in a treatment unit. In the study, the structure of bacteria community varies with phases of petroleum degradation. The study noted that genus *Flavobacterium* and *Pseudomonas* were dominant when degradation rate was high. This implies that microorganisms that start degradation may not be present by the end of the degradation process (Luo et al., 2009; Niepceron et

al., 2010). The reason for the disappearance was attributed to toxicity of the degradation product(s) on the degraders (Pelz et al., 1999). If combination of different strains are involved, some may be resistant to such toxic intermediates and help to remove them (Bobadilla Fazzini et al., 2010). Consortium of strains also help in co-metabolism (Nzila, 2013). No doubt, influence of inter- and intra-species is complex and it was profoundly reviewed by (McGenity et al., 2012). Nonetheless, some studies indicated that cyanobacteria were the primary colonizers after the gulf war spill (Al-Thukair and Al-Hinai, 1993).

2.4.2 Bacterial degradation and roles of other microorganisms

Even though bacterial degradation has been widely studied, (Abed and Köster, 2005; Abed et al., 2011; Kaplan and Kitts, 2004), reports have revealed presence of various species of cyanobacteria and diatoms in degradation activity site of microbial mats (Abed et al., 2006; Al-Thukair et al., 2007; Sánchez et al., 2005). Abed and Koster, (Abed and Köster, 2005) indicated degradation process is carried out by heterotrophic bacteria and not by cyanobacteria. However, cyanobacteria assist indirectly in degradation by supplying the oil-degrading heterotrophic bacteria with oxygen needed to breakdown aromatic and aliphatic compounds (Abed et al., 2002). They also supply ODMs with nutrients and enzymes, which are often the limiting factor on field.

In a study by (Sánchez et al., 2006), it was confirmed that cyanobacteria do not play direct role in the degradation of hydrocarbon because, they were not able to use hydrocarbon as

carbon or energy source. Microorganisms noted to contribute actively and directly were mainly alpha and gamma proteobacteria, chlorobi and low G+C gram-positive bacteria. Studies on isolation of specific bacterial strains in microbial community found in the gulf capable of degrading PAHs, were not found in literature.

Apart from alpha and gamma proteobacteria, (Abed et al., 2006) also detected beta and delta proteobacteria as well as Cytophaga-Flavobacterium-Bacteriodes group and Spirochetes after cloning bacteria from algal mat. About 15% of the detected strains were not described and suspected to be novel. Because of the high salinity and temperature of the Gulf area, their study assessed the influence of these factors in the degradation process and adaptability of the microbes in the harsh arid condition. They concluded that microbial mats from the region contain thermo-tolerant and halotolerant microbes that can degrade hydrocarbons even at elevated temperatures and salinities.

Like bacteria, fungi also utilize oil and PAHs as carbon and energy sources. Fungal degradation of oil and PAH have been extensively studied (Haritash and Kaushik, 2009; McGenity et al., 2012). Oil-degrading bacterial community benefits good supply of nutrients (especially N and P) if fungi are present. Fungi (*Actinomycetes*) are also renowned for production of bio-surfactants. Studies on oil-utilizing fungi in Arabian Gulf were reviewed (Hashem, 2007). It was noted that fungi has a weaker ability to degrade PAH when compared to bacteria. In fact, from 38 samples collected from the Kuwait coastal area of the Gulf, no high-temperature tolerant oil-degrading fungi were found among the 368 isolates (Sorkhoh et al., 1993). These indicate that bacteria is more relevant to biodegradation in the desert gulf.

2.5 Monitoring microbial diversity: Microscopic or molecular analysis?

The procedures of isolation, identification and characterization of microorganisms are tedious and multi-phasic. They can be grouped into microscopy and cultivation, molecular analysis, and phylogenetic analysis. Sometimes, only microscopy and one or few methods of molecular analysis are used for identification and classification. For instance, Al-Thukair et al. used light microscope to determine the genera of cyanobacteria present and dominant in their studied mats while they used molecular analysis by Denaturant Gradient Gel Electrophoresis (DGGE) of PCR-amplified 16S rRNA fragments to monitor the cyanobacteria diversity (Al-Thukair et al., 2007). Many research (Abed et al., 2006; Luo et al., 2009) are based on the use of one or combination of different methods of molecular analysis.

The procedures involved in nucleic acid extraction, RNA extraction and reverse transcription, PCR and DGGE was described vividly and shown to be reliable identification and diversity monitoring techniques (Abed and Garcia-Pichel, 2001; Al-Awadhi et al., 2012). When these procedures are combined with the use of light microscope and scanning electron microscope (SEM), they provide robust research methodology that can produce ground-breaking results.

CHAPTER 3

RESEARCH METHODOLOGY

3.1 Sample collection and characterization

The importance of proper sampling cannot be over-emphasized. If it is done carelessly, the whole results will be affected. Extreme care was therefore taking in sampling and we adhere strictly to all quality control and quality assurance protocol required for each of the experiments and procedures. The sediment samples were characterized for both physical and chemical parameters.

3.1.1 Sampling and sampling location

Sediment samples were collected from oil-polluted locations in coastal areas of Jubail industrial city (Lat. 27^{o}07'05"N; Long. 49^{o}34'16"E), Jubail, Saudi Arabia. The sampling locations were parts of the sites contaminated by oil spill from 1991 Gulf War. Three to five representative samples were taken from three different sampling points shown in Figure *3-1*. The first location point labeled A is Al-Nakheel Beach which is relatively cleaner than the other two locations B and C. There are obvious evidences of oil contamination at the two latter coastal locations designated as B and C. The contaminated soil/sediment samples were collected aseptically and stored in labelled sterile plastic plates with cover to preserve and protect loss of important members

of the microbial communities. Temperature and pH of the sampling points were measured where possible. Other physical and chemical parameters were measured in the lab.

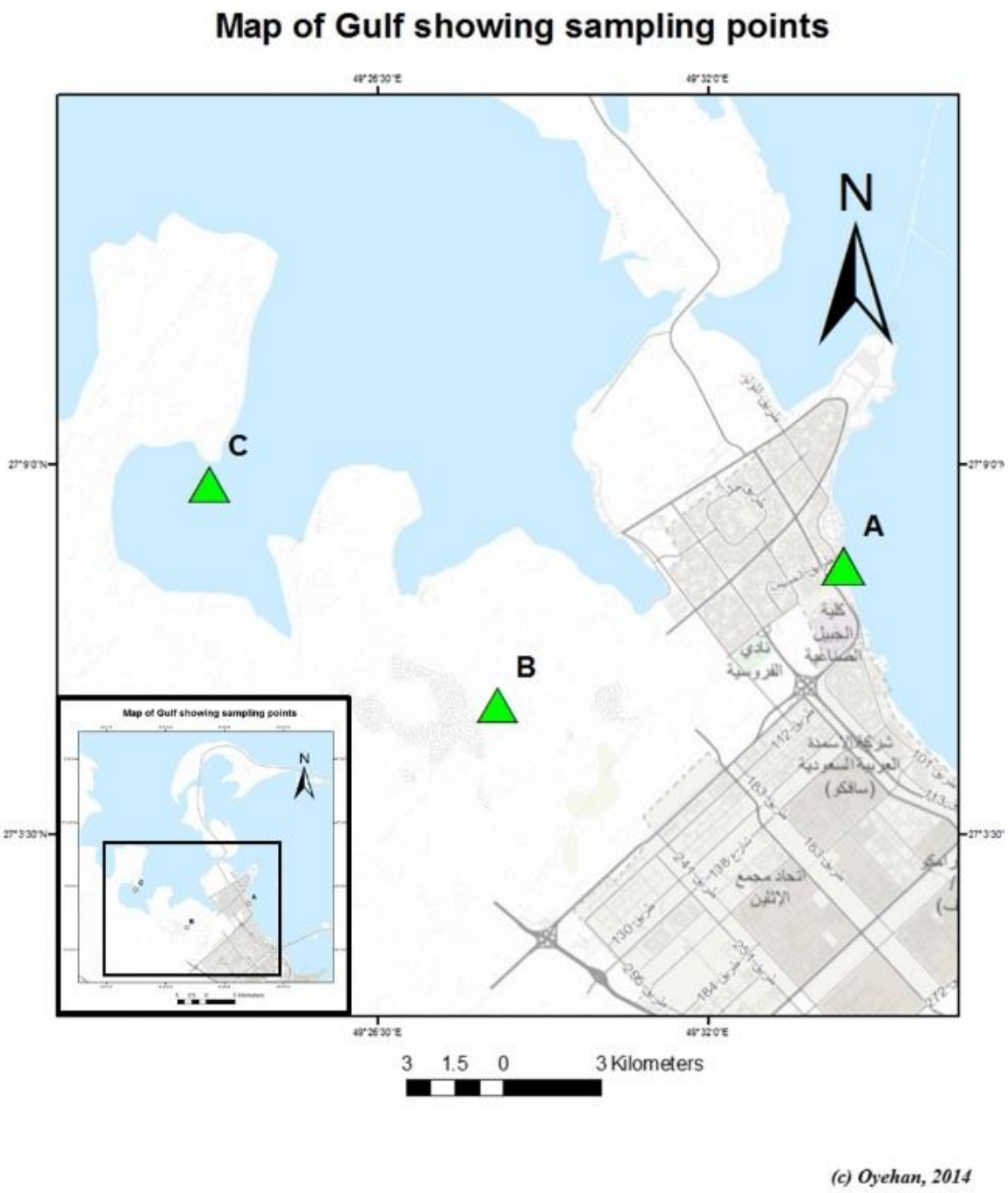


Figure 3-1. Map of Jubail, showing sampling locations A, B and C

3.1.2 Physical and chemical characterization of the samples

In the laboratory, the sediment samples were divided into portions. A portion was used for physicochemical parameters assessments (such as pH, salinity and ionic concentrations), another portion was air-dried, grinded and kept for TPH analyses. While the other portions were kept at low temperature for microbial experiments (enrichment and isolation).

3.1.2.1 Conductivity and pH assessments

Conductivity and pH values of the sediment samples were taken by using mechanical agitation soil extraction method. Adopted procedure involves extracting 20 g of soil with 40 ml of deionized (DI) water and stirring it for 5 minutes using magnetic stirrer. Electrical conductivity and pH values were then taken. The mixtures were filtered and the filtrates are kept for (ionic) chemical analyses in IC.

3.1.2.2 Nutrients (Ionic) analyses

Available soil nutrients in the sampling locations were analyzed with Ion Chromatography slightly modifying the (Jackson, 2000) procedures. Specifically, soil anions were analyzed with Dionex-Thermo ICS-3000 while cations (and anions again) were analyzed by Metrohm 850 Professional IC. Filtrate from soil-water extraction for pH and conductivity was used. However, due to the samples' large salinity values, the filtrates were diluted a hundred times. This dilution factor was taken into consideration while interpreting the IC results.

Instrument conditions for ICS-3000: The elution was gradient with KOH ranging from 10 Mm to 40 Mm of the eluent. The sample injection volume was 10 µl filled in a

loop of the same and injected into a Dionex ion Pac AS19 Column, and a guard column of ion Pac AG19 for separation. Suppression of the eluents conductivity was done by the Dionex AERS500 4 mm suppressor and the detection with Dionex conductivity detector. The calibration was run from 0.2mg/L to 100mg/L to give a linearity of 0.998, and then the samples were diluted to approximately come to calibration range.

3.1.2.3 Petroleum hydrocarbon

Extractable petroleum hydrocarbons (EPHs) in the sediment samples were analyzed using SiteLAB UVF-3100 (USEPA, 2001). Succinctly, the procedure begins with extraction of 5 g of soil in 10 ml of HPLC grade methanol. The mixture is shaken vigorously for minutes and then allowed to settle. The supernatant top layer is filtered and then diluted serially to ensure the concentration is within the instrument's calibration range. The diluted filtrate is measured in a fingerprint-free cuvette with SiteLAB UVF-3100.

3.2 Isolation and characterization of hydrocarbon-degrading bacteria

For selection of hydrocarbon-utilizing bacterial strains, Bushnell Haas (BH) liquid medium lacking carbon source was prepared and used for cultivation and enrichment. Selection of the hydrocarbon utilizing isolates was done on BH agar, while nutrient agar and broth were used to achieve quick growth of the isolates. Both solid (agar) and liquid (broth) culture media used during this research are described below;

3.2.1 Culture media

Bushnell Haas (BH) agar and BHA liquid medium were prepared following a similar procedures except that while liquid BH contain does not agar. One liter of the media contains 0.2g $MgSO_4$, 1g KH_2PO_4, 1g K_2HPO_4, 1g NH_4NO_3, 0.02g $CaCl_2$ and 0.05g of $FeCl_3$. In case of BHA, 15g of agar (Cole-Palmer® product) was added. The solution was made up to 1 liter with distilled water and sterilized in Astell® autoclave for 15 minutes at 121^{o}C. These culture media (liquid and solid media) were used for cultivation, enrichment and selection of hydrocarbon-utilizing bacterial strains, and for degradation experiments. Nutrient agar and broth used for growing and preserving the isolates were manufactured by Scharlau® Microbiology.

3.2.2 Enrichment and Isolation

Enrichments were carried out as described by (Madueño et al., 2011) with some modifications. Briefly, for isolation of diesel-degraders, 1.0 g of soil sample was suspended in 250-ml Erlenmeyer flask containing 100 ml of autoclaved BH liquid having 1% diesel as the only carbon and energy source. The culture was incubated for 7 days at 37^{o}C in a WiseCube Shaking incubator (WIS-20) set to 100 rpm shaking speed. Afterwards, 1.0 ml from the culture was then transferred to another 100 ml BH broth having 1% diesel and incubated under similar condition for another one week. This was repeated for three consecutive times to ensure selection of those that can

withstand imposed pressure. Similar procedure was repeated for phenanthrene and pyrene except that their concentration was 1000 mg/l.

After enrichments, isolation was done on BH agar plates on which specific PAH or diesel solution has been layered. Subsequently, colonies with distinct morphologies were selected and, then plated on NA plates. Isolated strains were purified by repeated sub-culturing and kept for identification and characterization. They were later prepared in agar slants and kept at very low temperature and, other two replicates were preserved under -20^{o}C in glycerol stock.

3.2.3 Characterization of the isolates

The isolates were characterized morphologically, biochemically, phylogenetically and by their ability to degrade specific hydrocarbons. Hereunder however, the characterization were aimed at identifying the strains' broad group. Therefore, the central focus was phenotypical characterization (i.e. the morphology and biochemical behavior).

3.2.3.1 Morphological characterization

Colony and cellular morphologies were studied. The isolates were streaked on minimum nutrient NA plates. Minimum nutrient was used, to ensure forming of clear, easy-to-observe colonies. The colonies' form, elevation and margin were observed under dissecting microscope. Cellular morphology (shape) was observed in light microscope after heat-fixing a 24-hour incubated freshly grown isolate on a glass slide and their

micrographs were taken with Zeiss Imager.D2 compound light microscope equipped with AxioCam MRc camera and AxioVision software.

3.2.3.2 Biochemical characterization

The isolates are tested for their reaction to Gram's staining after heat-fixing to glass slides. Briefly, the procedure involves smearing a very small amount of 24-hour grown culture on a glass slide, heat-fixing and staining it. To stain, the slide is flooded for 1 minute with crystal violet and then washed with distilled water. This is repeated with iodine solution. To decolorize the strains, 95% ethanol was used to wash it for 25 – 35 seconds. Counterstaining is done with Safranin. The slides are drained and observed in microscopes.

The ability of the isolates to utilize different hydrocarbons (diesel, pyrene, phenanthrene and anthracene, and a combination of the three PAHs) as carbon and energy source was tested on BHA solid medium applying the technique of agarose overlay (Madueño et al., 2011). On the plates, few circles were drawn on the plate to estimate the spread of the isolate cells/colony as they grow (utilize) the specific hydrocarbon. The plates were incubated at 37^{o}C and growth was monitored after 5 – 7 days.

3.2.4 Scanning Electron Microscope

Isolated bacterial sample was prepared for SEM examination according to the method described by (Piroeva et al., 2013) with little modification regarding accelerating voltage wherein we used 20 – 30kV that we found to be best as against the prescribed

15kV. Briefly, 0.8% agar-water solution was prepared and heated to about 50 to 60^{o}C. Then, clean glass cover slides previously sterilized in UV for 10 to 15mins are then dipped in the tepid agar-solution, brought out and kept in horizontal position. After few minutes, 20 µl of isolate solution is pipetted onto the thin agar film formed on the slide. The isolate solution is made from 12-hour nutrient-broth-grown strains that is centrifuged and then made into very dilute solution with distilled water. The slides containing bacterial solution on thin agar film are left for 30 mins and then dehydrated in oven at 37^{o}C for 12 hours. The embedded samples are fixed and further dehydrated using traditional method of serialized dipping sample in increasing alcohol concentration. In this case, the ethanol concentration (in percent) was in other of 10, 25, 50, 75, 96 and, then absolute. The samples stay a minimum of 30 minutes in each concentration. The slides were placed in oven at 37^{o}C for 90 minutes for final drying. They were then glued to stub and coated with gold for examination in JEOL JSM-6460LV Scanning Electron Microscope.

3.3 Molecular analyses and Identification of the isolates

The pure isolates were identified by sequencing their respective 16S rRNA which is the most conserved DNA region in prokaryotes. The evolutionary history and relationships of the isolates were evaluated by constructing phylogenetic trees for them using consensus DNA sequences.

3.3.1 16S rRNA sequencing

Pure representative isolates was suspended in a 0.5 ml of sterilized saline and centrifuge at 10,000 rpm for 10 mins. The pellet obtained was then suspended in 0.5 ml of InstaGene Matrix (Bio-Rad, USA) and incubated at 56^{o}C for 30 minutes. The suspension was then heated to 100oC for 10 minutes. The supernatant containing soluble DNA was used as template for PCR.

The PCR amplification of 16S rRNA genes were performed in 35 cycles, using reference (*E. coli*) primers 27F (5V–AGA GTT TGA TCC TGG CTC AG–3V) and 1492R (5V–TAC GGY TAC CTT GTT ACG ACT T–3V) in the first cycle for bacteria at PCR conditions of 94^{o}C for 45 sec, 55^{o}C for 60 sec and 72^{o}C for 60 sec (Luo et al., 2009). The bacterial DNA fragments are amplified at about 1,400 bp. *Escherichia coli* was used as positive control. The amplified 16S r RNA was purified by removing unincorporated PCR primers and dNTPs using Montage PCR Clean up kit (Millipore).

The pure amplified PCR products of about 1,400 bp were sequenced using forward bacterial primer 518F (5V–CCA GCA GCC GCG GTA ATA CG–3V) and reverse primer 800R (5V–TAC CAG GGT ATC TAA TCC–3V). The sequencing were performed using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA) and resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA). The obtained sequences were submitted to National Center for Biotechnology Information (NCBI) basic local alignment search tool (BLAST) program for comparison with those in the GenBank database. The isolates' sequences have been deposited into GenBank database as pure strains with unique accession numbers.

3.3.2 Gel Electrophoresis

The lengths of the isolates' DNA were determined by gel electrophoresis. The procedure was performed as described by (Al-Awadhi et al., 2012). Briefly, aliquots of the PCR amplification products were run on a freshly prepared agarose gel (previously stained with ethidium bromide for visualization) at 80 – 150V until the dye line is approximately 75 – 80% of the way down the gel. The power source is turned off and the DNA fragments in the gel is visualized using UV transilluminator.

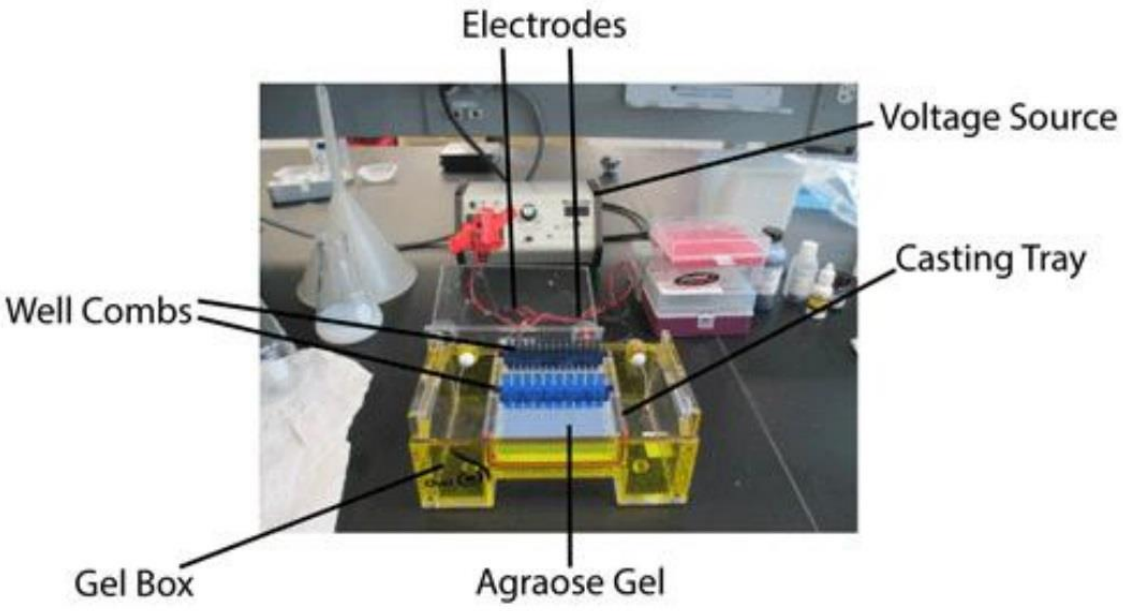


Figure 3-2. Gel Box and parts

3.3.3 Phylogenetic analysis

Phylogenetic trees were constructed using the Molecular Evolutionary Genetics Analysis (MEGA version 6.0) software package (Tamura et al., 2013). Consensus sequence of forward and reverse sequences of the 16S rDNA were obtained by aligning them with Java program Sequlator®, and Bioedit® software. The consensus sequences of the 16S rRNA genes with more than 1400 bp and sequences of related bacteria from BLAST program were aligned using the online web server alignment and tree construction program called Phylogeny.fr, (Dereeper et al., 2008).

The alignment results were used to construct phylogenetic trees in MEGA 6.0 and their evolutionary history was inferred by neighbor-joining method (Saitou and Nei, 1987). The strength of the phylogeny was evaluated using bootstrap test with 500 replicates or iterations (Felsenstein, 1985). Kimura 2-parameter method (Kimura, 1980) was used to compute the evolutionary distances in the unit of the number of base substitutions per site. Variation rate among sites was based on gamma distribution model (with shape parameter = 2). Our analyses phylogenetic trees involved 13 or more nucleotide sequences. Gaps and missing data within the sequences were eliminated.

3.4 Biodegradation assays

The inocula of bacterial strains BC1, BC5, BC6, BC7, DSA, LA and LB were prepared for biodegradation experiments as described by (Abed et al., 2014) with little modification. In brief, 12-hour freshly grown isolates in NA broth were concentrated by centrifugation and

then washed three times. The pellet was re-suspended in about 2 ml BH medium to make a concentrated bacterial solution. These selected isolates were chosen based on a number of factors which are: (i). Unique morphology, e.g. BC1 and LB; (ii) Overall performance in utilizing different hydrocarbon based on growth on agarose overlay (see section 3.2.3.2 above). (iii) Isolation source e.g. DSA and, (iv) Uniqueness of identity e.g. LA, LB and BC1.

3.4.1 Diesel degradation

Degradation of diesel was assessed in 250-ml conical flasks containing 100-ml culture of 98% BH liquid, 1% diesel as carbon source and 1 ml of inoculum suspension. The cultures were incubated at 37^{o}C on a shaker at 100 rpm. Colony count of the strain is carried out at the outset of the experiment and at regular interval. OD and diesel concentrations were measured with UV-VIS spectrophotometer at 600 nm wavelength, and GCMS respectively from day 1 up to day 30. The setups with controls are made in triplicates. Control cultures have similar composition except lack of inoculum.

3.4.2 PAH (phenanthrene and pyrene)

Setup for PAH degradation experiments is similar to that of diesel degradation described above. The difference is in the concentration of the hydrocarbon. Acetone was used as solvent to dissolve the PAH (pyrene and phenanthrene) at a concentration of 10g/l.

From the PAH solution, 1 ml was pipetted into sterilized empty 250-ml conical flask and made up to 99-ml mark with autoclaved BH liquid. The final PAH concentration in the culture was 100ppm. The 99 ml culture was left in the shaker overnight to allow acetone to escape. Then, 1 ml inoculum was added and incubated at 37^{o}C in shaker at 100 rpm. Colony count, pH, OD and concentrations were taken from day 1 and every two days up to day 15. Similar set up with no inoculum was used as control. Both test and control experiments were in three replicates.

To assess performance of enrichment culture containing mixed bacteria against pure isolate, 1 ml of liquid enrichment culture from the flask from which strain BC1 was isolated was used as inoculum for pyrene degradation. BC1 was also used as inoculum in another flask containing equal pyrene concentration. At day 1, 3, 10 and 40, concentration of pyrene in the cultures were analyzed with GCMS to determine their degradation pattern. All experiments were in triplicates

3.5 Analytical chemistry

All chemicals used in the experiments were of HPLC grade and from reliable commercial sources. The same GC conditions were maintained throughout the analyses. GC method adopted for each of the PAHs was the same but differ from that used for diesel. SPME extraction time was also varied due to difference in volatility and other physical properties of diesel and PAHs.

3.5.1 Chemicals and their sources

Pyrene and phenanthrene were both supplied by Sigma-Aldrich® and were at purity of 98%. While anthracene was supplied by MERCK® as HPLC grade at >96% purity. Acetone used as solvent for dissolution of the PAHs was from Fluka® with 99.5% purity (GC assay). During the extraction of EPH from the sediment samples, methanol used was from MERCK® and at 99.8% purity (GC assay). Ethanol used for embedding and dehydration of isolates for SEM analyses was HPLC grade at 99.8% purity and supplied by Fluka®.

3.5.2 GC-MS with SPME

Extraction of residual hydrocarbon, at the start of the experiment and during subsequent assessments, in culture was done with solid phase micro-extraction (SPME) Figure *3-3*. The SPME non-polar fiber is made of polydimethyl siloxane (PDMS) with 60 mm thickness. Unlike in conventional degradation assay method where batches of cultures are sacrificed, only 2 ml of the liquid is pipetted after shaking very well into GC vial. Extraction is then carried out from the small volume of the culture. This is repeated at regularly at all sampling intervals. For diesel, the fiber is inserted through its holder into the whole culture as described by (Eriksson et al., 1998). After extraction the fiber is injected directly into GC injection port.

The 6890N Network GC System manufactured by Agilent Technologies was used throughout the experiment. It was fitted with mass spectrometry detector (MSD).

The GC column is coated with HP-5MS 5% phenylmethyl siloxane film and its dimension is 30m x 250µm x 0.25µm. Carrier gas used is helium at 99.99999% purity. There are some differences in the GC methods and SPME condition used for PAHs and diesel.

Figure 3-3. SPME holder

3.5.2.1 GC methods and SPME extraction condition for diesel

Extraction time used for diesel degradation assay culture is 2 minutes. After extraction, the fiber is injected manually into the GC column at a temperature of 220^{o}C. The 30 meter long GC capillary column is lined with 0.25 µm thickness film of HP-5MS 5% phenylmethyl siloxane and has internal diameter of 250 µm.

GC analytical method used was as described by (Eriksson et al., 1998) in a similar study. Briefly, initial oven temperature was programmed at 40^{o}C, held for 4 minutes and ramped to 120^{o}C at 4^{o}C/min and then at 10^{o}C/min up to 200^{o}C. Oven temperature was held at 200^{o}C for 11 minutes. The MS detection temperature was set at 250^{o}C. The carrier gas used throughout the analyses was helium flowing at 1.5 ml/min. After each run, column

was cleaned by post running at 280^{o}C holding it for 2 minutes. While the SPME fiber is also conditioned (cleaned and prepared) for subsequent use by first leaving it in the injection port for 5 minutes to burn out any hydrocarbon remnant on the fiber. The fiber is then run blank at a higher injection temperature to confirm that it is truly free of the analyte.

3.5.2.2 GC method and SPME extraction condition for pyrene and phenanthrene

Because PAHs are less volatile than diesel components, extraction time was longer and agitation was applied with the use of a small magnetic stirrer to increase extraction efficiency (King et al., 2004). For consistency, the chosen extraction time of 20 minutes and agitation speed were not varied. After extraction, the fiber was injected into GC and left in the port for 10mins for complete desorption. The SPME was conditioned in the GC injection set at 300^{o}C and run blank, before and after each analysis.

Extracted phenanthrene on SPME fiber was desorbed into GC injector port at 220^{o}C and left there for 7 minutes. GC oven temperature was set to and held at 50^{o}C for 2 minutes, and up to 200^{o}C at 15^{o}C/min and finally at 7.5^{o}C/min to 260 and held for 2 minutes. Carrier gas is 99.9999% helium gas flowing 23.3 mL/min in a splitless mode at pressure of 0.69 psi. The column is purged through split vent at 20 mL/min flow. For pyrene, the conditions are similar except that GC oven is ramped from 50 to 150oC at 10oC/min and then at 5oC/min to 300^{o}C holding it for 5 minutes before post-running at 320^{o}C.

Estimation of degradation was based on a similar formula applied by (Wu et al., 2013) where;

"*Percentage of hydrocarbon degraded was estimated as the ratio of the difference between initial HC concentration (C_0) and final concentration (Cf) to initial HC concentration expressed as a percentage"*. That is,

$$\%\ degraded = \frac{\mathrm{Co} - Cf}{Cf} * 100\%$$

3.6 Statistical analysis

Data from the experiments were summarized in Microsoft Excel® and analyzed (ANOVA and correlations) using SigmaPlot® version 11.0. Charts and graphs were drawn with Excel and SigmaPlot.

Molecular analysis involving G+C percent estimation was computed with an in-built statistical function in Molecular Evolutionary Genetics Analysis (MEGA) version 6.0 (Tamura et al., 2013).

CHAPTER 4

RESULTS

4.1 Physicochemical characterization of the sediment samples

Physicochemical analyses of the sediment samples collected from the three different coastal locations in Jubail area of Arabian Gulf show that the soils' pH values range between 8.34 – 9.80 and concentrations of extractable petroleum aromatic hydrocarbons between 9.09 – 13.42 mg/Kg (Table *4-1*). Location A, the less polluted Nakheel beach (EPAH = 9.09 mg/Kg), showed a higher pH value of 9.8 (i.e more alkaline than others). Sampling points in this location is frequently washed over by neap (slow) tide, unlike in other locations where the water is practically stagnant.

Ionic concentrations of the two more polluted locations (locations B and C) are generally higher than that of relatively clean location A (Nakheel beach). Nevertheless, for all locations, nitrogen is present in small amount and mainly as nitrates (31.6 to 60.8 ppm) while phosphorus which is equally very important to the survival and proliferation of microorganisms is detected at very low concentration (Table *4-1*).

Concentration of individual anions was correlated with that of cations in order to use their relationships to depict how the ions co-exist as compounds. It is observed that most of cations correlate strongly with anions as expected except for nitrate and bromide (Table *4-2*). While nitrate shows no or weak relationship with other ions, bromide shows

association with only potassium (r^2 = 0.83) and magnesium (r^2 = 0.912). Sodium (0.999) and potassium (0.902) occur mostly as NaCl and K_2SO_4 salts respectively. And sulfate is present mainly as $CaSO_4$ (0.979). The import of these is that the soils are rich in some essential nutrients (K, Ca, Mg, etc.) required for success of microbes, but low quantities of N and P might be limiting their productivity.

Table 4-1. Summary of soil physicochemical properties of the three sampling locations

Parameter* ***(in mg/Kg)***	**Location (Mean ± Std Error)**			**Remarks** ***(Sig. diff. is based on Fisher LSD)***
	A	**B**	**C**	
PAH	9.09 ± 1.66	12.27 ± 2.96	13.42 ± 4.04	NSD***
pH	9.80 ± 1.26	8.34 ± 0.72	8.63 ± 0.24	SD** (A)
Conductivity ***(mS/cm)***	7.21 ± 3.21	45.64 ± 17.5	21.49 ± 13.98	NSD
Chlorine	5610 ± 594	43564 ± 9723	19356 ± 3346	SD (A)
Bromine	107.0 ± 17.6	93.8 ± 19.3	106.4 ± 17.2	NSD
Nitrate	44.79 ± 3.66	60.76 ± 6.92	31.61 ± 12.38	SD (C)
Nitrite	< 0.02	< 0.02	< 0.02	
Phosphate	< 0.05	< 0.05	< 0.05	
Sulfate	695.7 ± 182.7	12783.4 ± 1955.7	6280.1 ± 1797.0	SD (all).
Fluorine	1.27 ± 0.44	1.00 ± 0.40	2.7 ± 0.93	NSD
Sodium	2841.05 ± 430.6	22513.27 ± 8860.9	9084.15 ± 2549.62	NSD
Potassium	91.30 ± 17.22	524.87 ± 58.09	373.45 ± 93.11	SD (A)
Magnesium	386.1 ± 68.9	1503.6 ± 221.2	1292.0 ± 288.9	SD (A)
Calcium	237.50 ± 9.23	2572.40 ± 250.21	1419.45 ± 309.12	SD (all)

*Mean values.

SD** implies statistically significant difference with p-value > 0.05 at confidence level of 95%.

NSD***, no significant difference at alpha value of 0.05.

Parenthesis *()*, shows where the observed difference exists.

Table 4-2. Correlation[1] table gives insight to how the ions might exist in the sampling areas

	Na^+	K^+	Mg^{2+}	Ca^{2+}	Cl^-	Br^-	NO_3^-	SO_4^{2-}
Sodium	1	0.8	0.801	0.768	0.999	0.515*	0.637	0.853
Potassium		1	0.983	0.834	0.823	0.83	0.612	0.902
Magnesium			1	0.803	0.827	0.912	0.541*	0.869
Calcium				1	0.784	0.429*	0.480*	0.979
Chloride					1	0.551*	0.63	0.868
Bromide						1	0.194*	0.527*
Nitrate							1	0.576*
Sulfate								1

*, no significant relationship at probability level of 0.05

[1], Correlation coefficients are calculated based on *Pearson Product Moment Correlation*

4.2 Microbial characterization

A total of 15 morphologically-distinct hydrocarbon-degrading strains were isolated from enrichment cultures of the sediment samples with specific hydrocarbon as sole-carbon source (Table *4-3*). The isolates were described and characterized by their morphologies and efficiencies in utilization of certain specific hydrocarbons.

4.2.1 Isolates and their sources

Enrichments on single PAH (phenanthrene, anthracene or pyrene) as sole carbon source yielded 10 strains (isolates BC1 – BC10) out of the 15 isolated hydrocarbon-degrading bacterial strains (Table *4-3*). In other words, 3 strains were isolated from each of phenanthrene- and pyrene-rich enrichment cultures while 4 isolates were from anthracene-rich broth. At least three distinct strains were isolated from each of the three sampling locations (Figure *4-1*). Enrichment culture of pyrene could not yield any isolate from location A sample. In contrast, out of the four isolates from location B, two were from pyrene-culture (one of which these two, labelled BC1, is morphologically distinct from all the isolates). Similarly, enrichment cultures of sample from location C yield one strain for each of the three PAHs used as sole carbon source; strains BC3 (Figure *4-4*) from phenanthrene enrichment culture, BC5 (Figure *4-6*) from anthracene and BC9 (Figure *4-10*) from pyrene.

Aside using single PAH as sole carbon source, enrichment cultures containing a combination of the three PAHs as carbon yielded one morphologically dominant strain

from each of the 3 sampling locations (Figure *4-14*, Figure *4-15* and Figure *4-16*). In addition to this, using diesel oil as enrichment carbon source, two diesel-degrading strains, DSA (Figure *4-12*) and DSB (Figure *4-13*) were further isolated from a composite of soil samples from locations B and C.

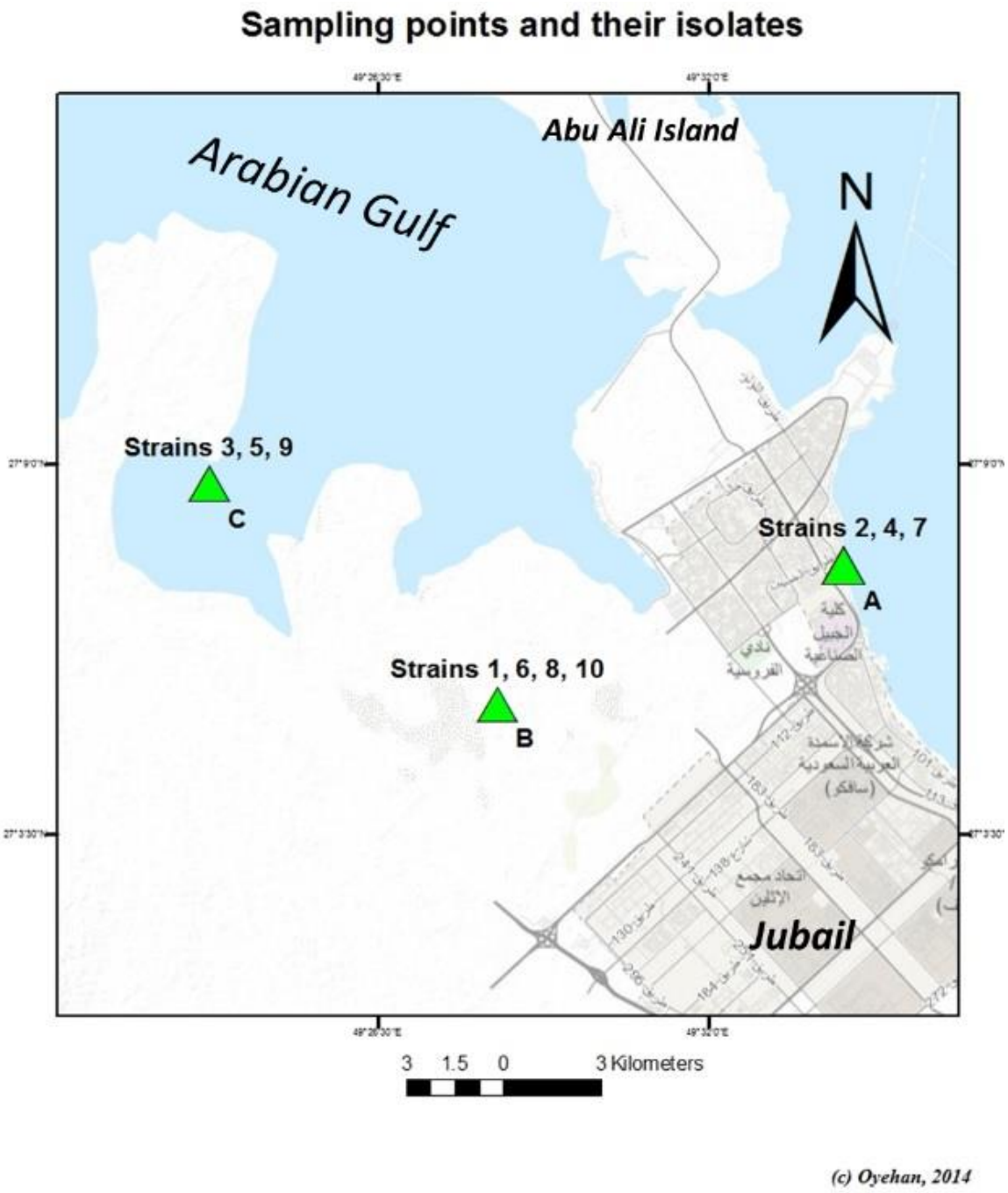


Figure 4-1. Map of sampling locations and their specific PAH-degrading isolates

Table 4-3. The fifteen isolates, the specific hydrocarbon (carbon source) used in enrichment cultures and their accession numbers

Strain label	Source Location	Carbon-source	GenBank accession no.
JBL_BC1	B	Pyrene	KP792293
JBL_BC2	A	Anthracene	KP662550
JBL_BC3	C	Phenanthrene	KP662548
JBL_BC4	A	Phenanthrene	KP662547
JBL_BC5	C	Anthracene	KP792289
JBL_BC6	B	Phenanthrene	KP662549
JBL_BC7	A	Anthracene	KP792290
JBL_BC8	B	Pyrene	KP792291
JBL_BC9	C	Pyrene	KP662551
JBL_BC10	B	Anthracene	KP792292
JBL_DSA-01	B&C	Diesel	KP683357
JBL_DSB	B&C	Diesel	KP792288
JBL_LA	A	Phe, Pyr, Anth	KP792294
JBL_LB	B	Phe, Pyr, Anth	KP792286
JBL_LC	C	Phe, Pyr, Anth	KP792287

4.2.2 Utilization of different hydrocarbon substrates

The 10 pure strains obtained from single PAH enrichments were grown on five different hydrocarbon substrates to assess and rank their ability to utilize them. Notably, all the strains grew, at varying time and luxuriance, on all the substrates except for BC4 which failed to utilize or grow on diesel.

By quantifying their growths on a small circle pre-drawn on the plates, we are able to rate and rank them (Table 4-4). Isolate BC5 (Figure *4-6*) is rated the best. It grew luxuriantly on phenanthrene, spread very well on pyrene and anthracene, and has good growths on a mixture of the three PAHs as well as diesel. Isolates BC6 (Figure *4-7*), BC7 (Figure *4-8*) and BC10 (Figure *4-11*) were also ranked good, therefore the former two were selected together with BC5 for further degradation assays. These strains are selected as a representative of the location from which they were isolated (see Figure *4-1*). Phenanthrene and anthracene were also found to be utilized more readily by the isolates than pyrene and a combination of all.

Table 4-4. Strains performance based on growth in BH plates with specific hydrocarbon

Isolate Label	**Substrate hydrocarbon**					**TOTAL**
	Phenanthrene	Pyrene	Anthracene	PAH	Diesel	
BC1	3	3	2	2	2	**12**
BC2	3	2	3	1	1	**10**
BC3	3	2	3	3	1	**13**
BC4	1	3	2	1	0	**08**
BC5	5	4	4	3	3	**19**
BC6	4	2	3	1	5	**15**
BC7	2	4	2	1	5	**14**
BC8	2	3	3	3	1	**12**
BC9	3	2	3	3	1	**12**
BC10	3	1	4	3	2	**14**
TOTAL	**29**	**27**	**29**	**21**	**21**	**127**

Growth was monitored by estimating spread of the colonies of inoculated strains within a pre-drawn circle on the plate. If the spread covers the whole circle, it is rated excellent (5), covers almost all is rated very good (4) and so on.

Excellent growth = 5; Very good growth = 4; Good growth = 3; Fair growth = 2; Poor growth = 1; No growth = 0

4.2.3 Morphologies of the isolates

The isolates were grown on nutrients agar (NA) plates, then a single colony of each strain was prepared on glass slides observed under microscope to reveal colony and cell morphologies respectively. Morphologies of single colony of the isolates prepared on glass slide indicated that all the 15 isolates are rod-shaped bacteria strains. Table *4-5* and Figure *4-2* to Figure *4-16* show the colony morphologies of the all the isolates as they appear nutrients agar (NA) plates and the micrographs of their respective single colony. Biochemically, all the isolates are gram negative bacteria.

Some of the strains were selected for biodegradation experiments. Morphologies of these strains were further observed under scanning electron microscope (SEM) to confirm their shapes and reveal their sizes (see Figure *4-17* - Figure 4-20 and, Table 4-6).

Table 4-5. Colony morphology of the fifteen isolates

Strain label	Form	Elevation	Margin
JBL_BC1	Irregular	Raised	Undulate
JBL_BC2	Irregular	Raised	Undulate(few entire)
JBL_BC3	Irregular	Convex/ Pulvinate	Entire
JBL_BC4	Circular	Raised	Entire
JBL_BC5	Filamentous	Umbonate	Lobate
JBL_BC6	Filamentous	Raised	Undulate
JBL_BC7	Irregular	Convex	Undulate
JBL_BC8	Circular	Raised	Entire
JBL_BC9	Filamentous	Crateriform	Filiform
JBL_BC10	Circular	Raised	Entire
JBL_DSA-01	Circular	Raised	Entire
JBL_DSB	Irregular	Raised	Undulate
JBL_LA	Filamentous	Flat	Undulate
JBL_LB	Circular	Convex	Entire
JBL_LC	Irregular	Raised	Undulate

Table 4-6. Cell size of two of the isolates from SEM analysis

Isolate	Length in µm (Mean ± SD)	Width in nm (Mean ± SD)
BC1 (*Ochrobactrum intermedium)*	1.388 ± 0.286	352.7 ± 5.8
BC6 (*Pseudomonas aeruginosa*)	1.400 ± 0.123	441.2 ± 0

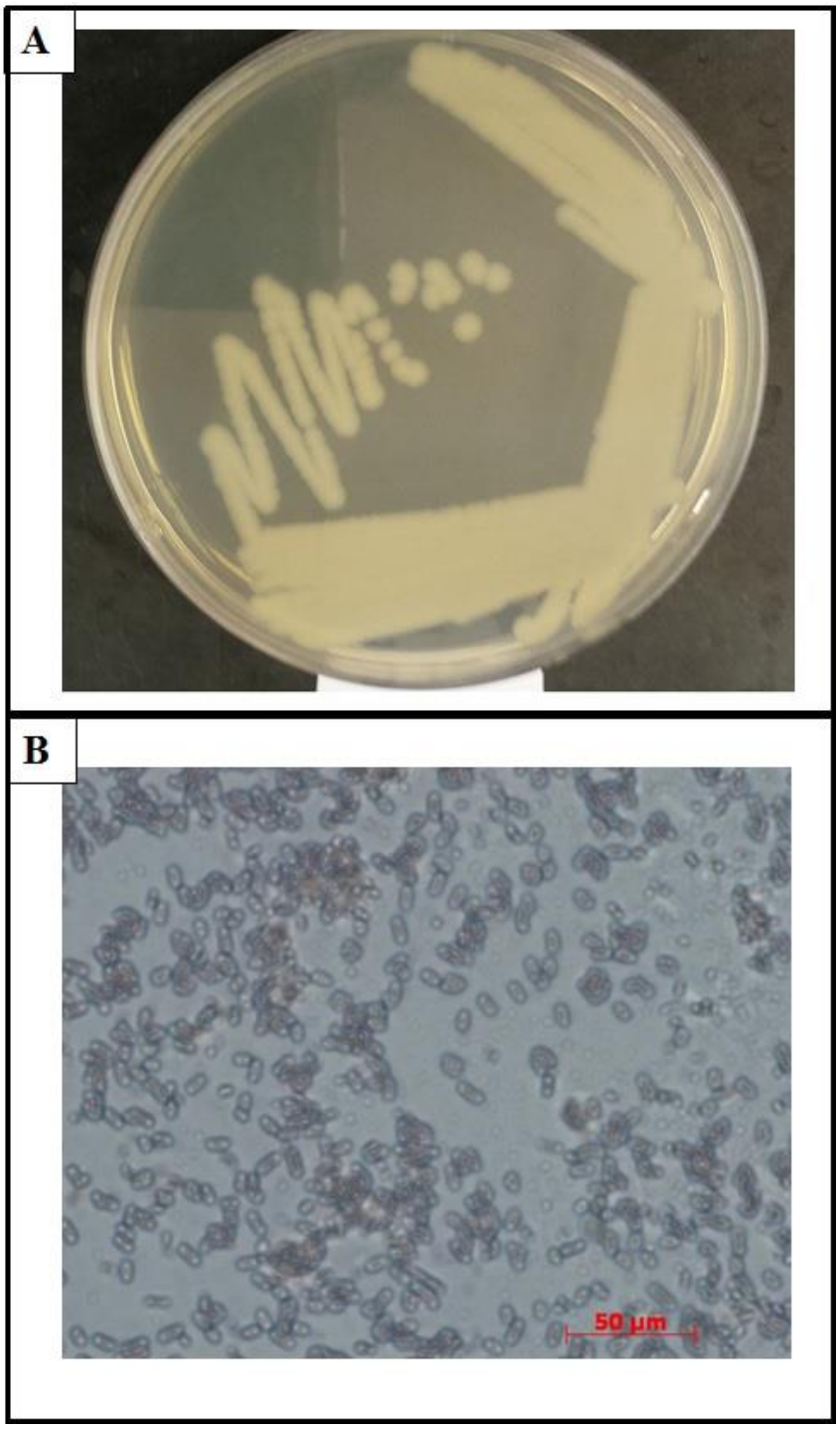


Figure 4-2. (A) Picture showing isolate BC1 colony morphology on NA plate. (B) Micrograph of isolate BC1 showing shape in a single colony (Mag. X100).

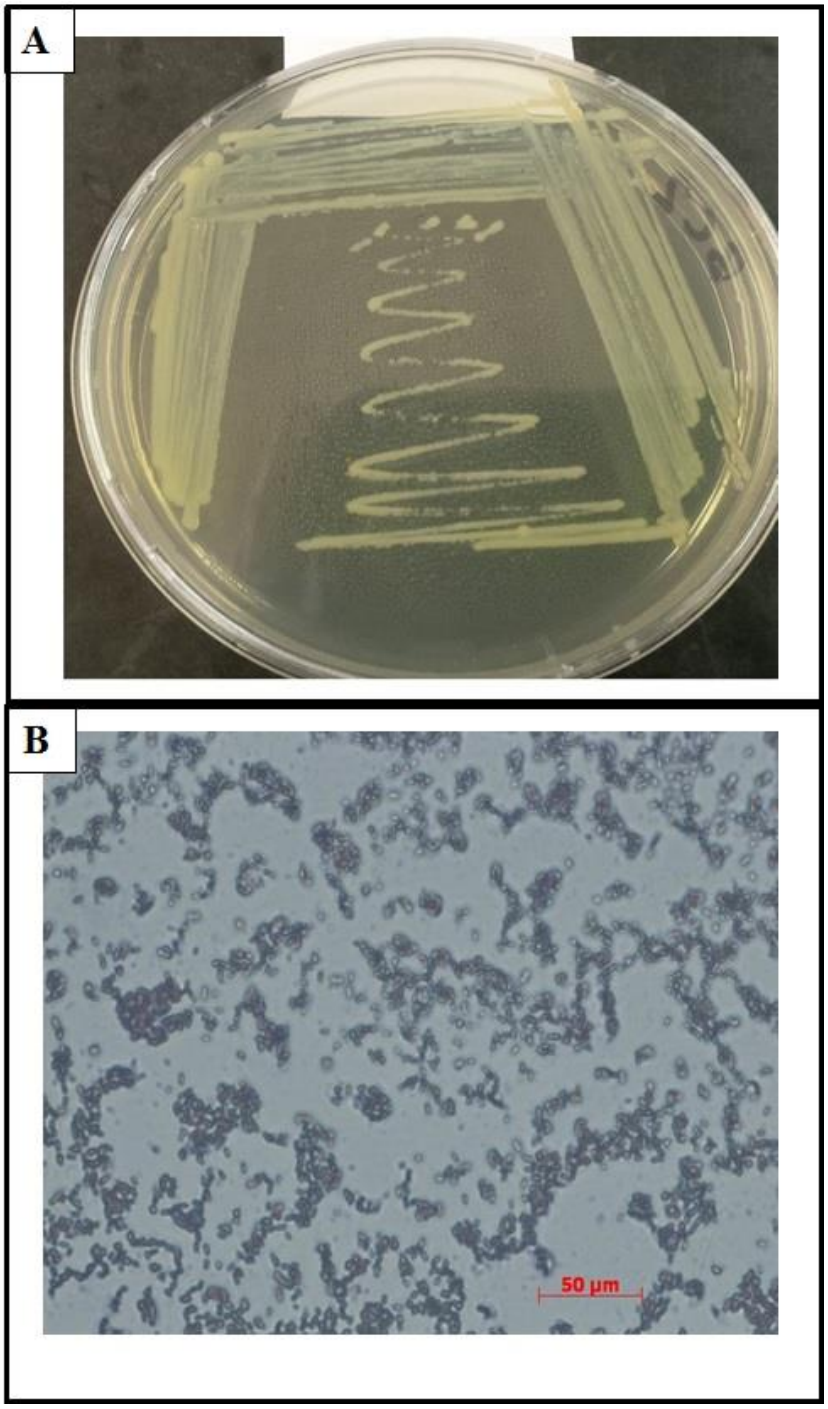


Figure 4-3. (A) Picture showing isolate BC2 colony morphology on NA plate. (B) Micrograph of isolate BC2 showing shape in a single colony (Mag. X100)

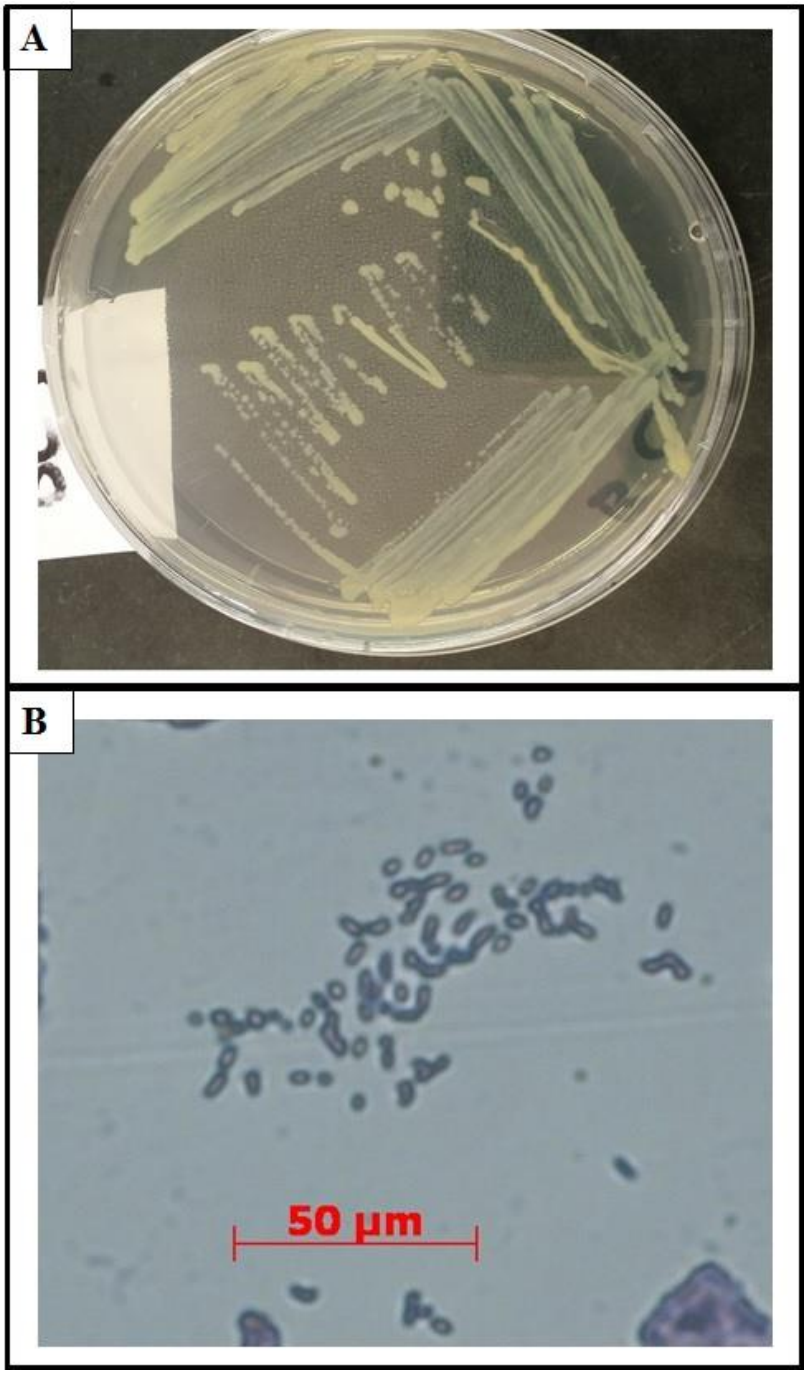


Figure 4-4. (A) Picture showing isolate BC3 colony morphology on NA plate. (B) Micrograph of isolate BC3 showing shape in a single colony (Mag. X100)

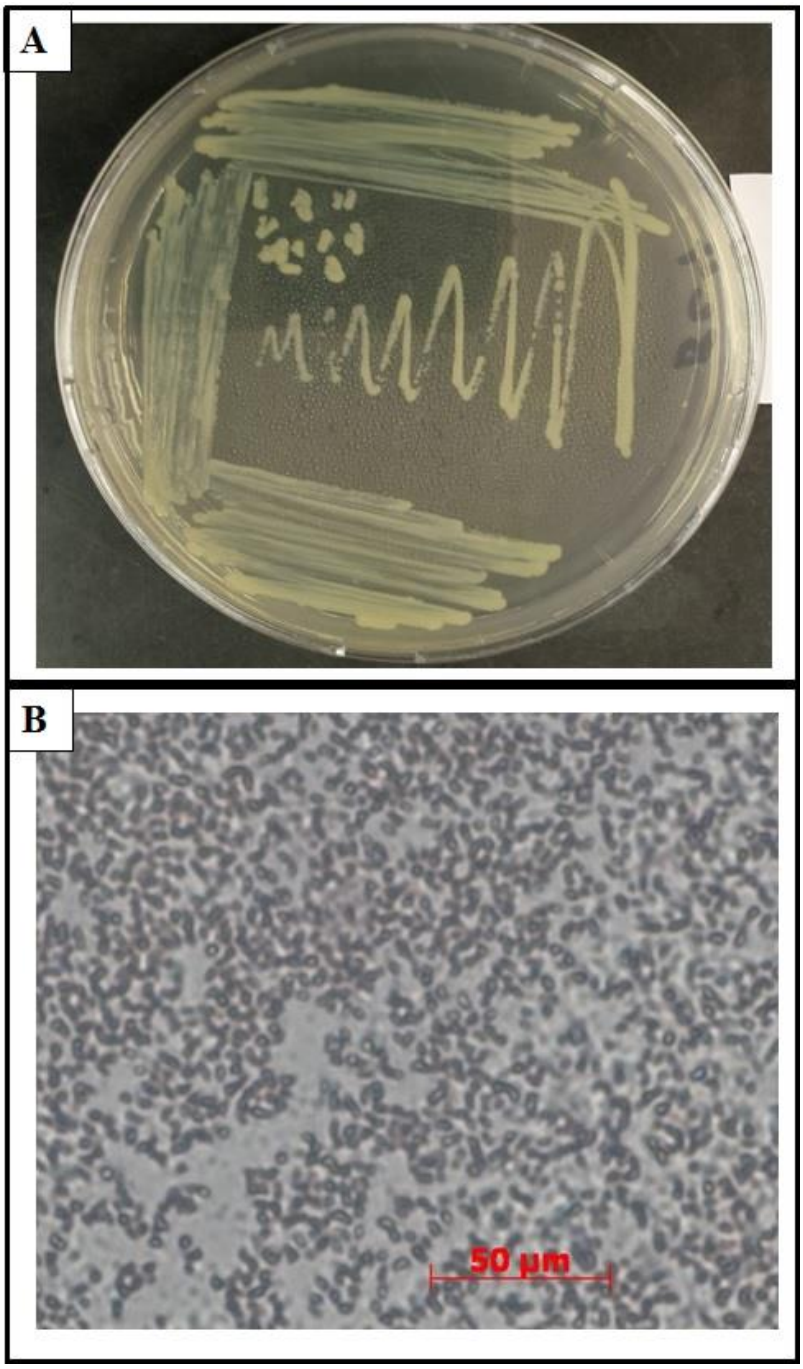


Figure 4-5. (A) Picture showing isolate BC4 colony morphology on NA plate. (B) Micrograph of isolate BC4 showing shape in a single colony (Mag. X100)

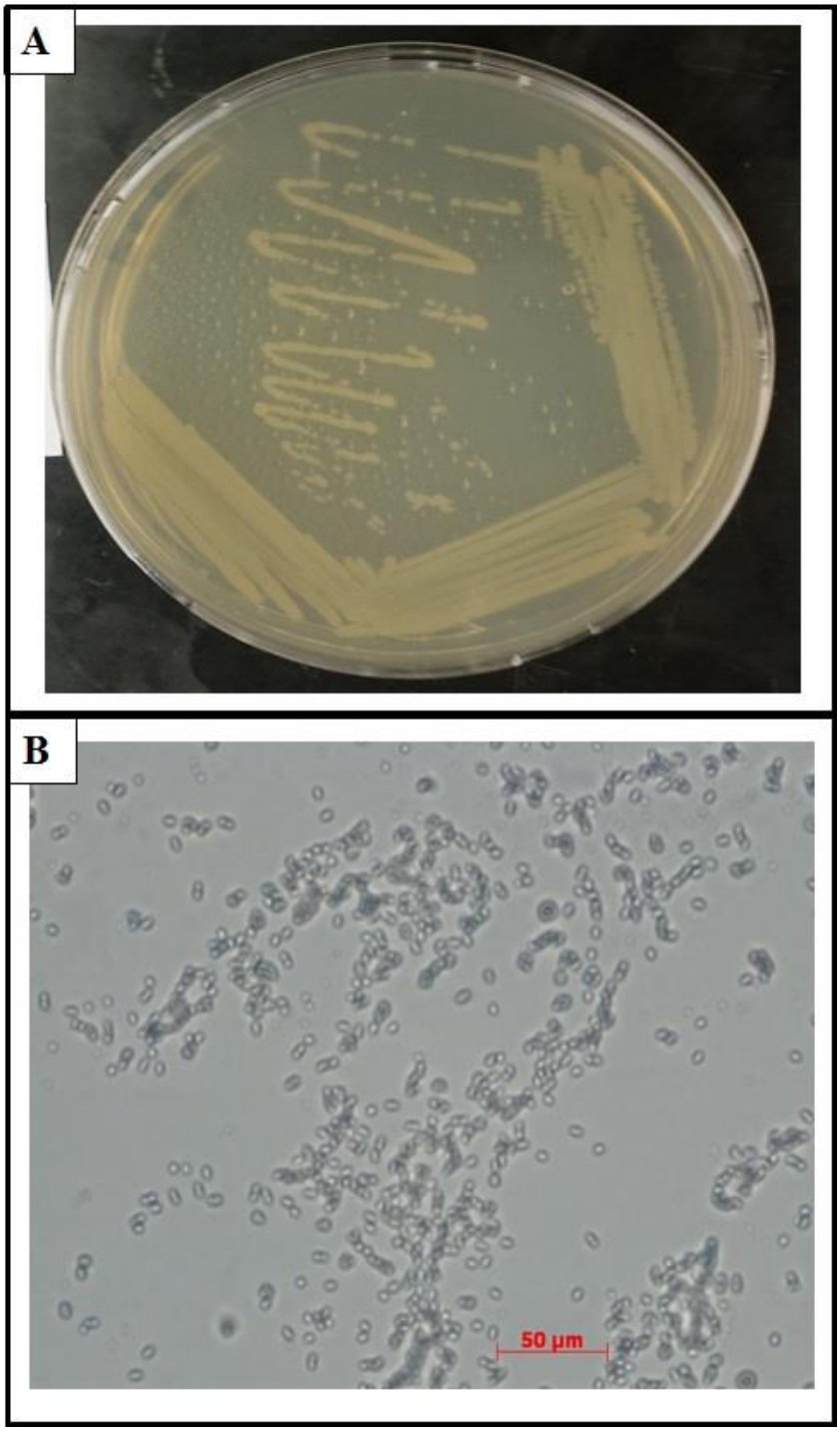


Figure 4-6. (A) Picture showing isolate BC5 colony morphology on NA plate. (B) Micrograph of isolate BC5 showing shape in a single colony (Mag. X100)

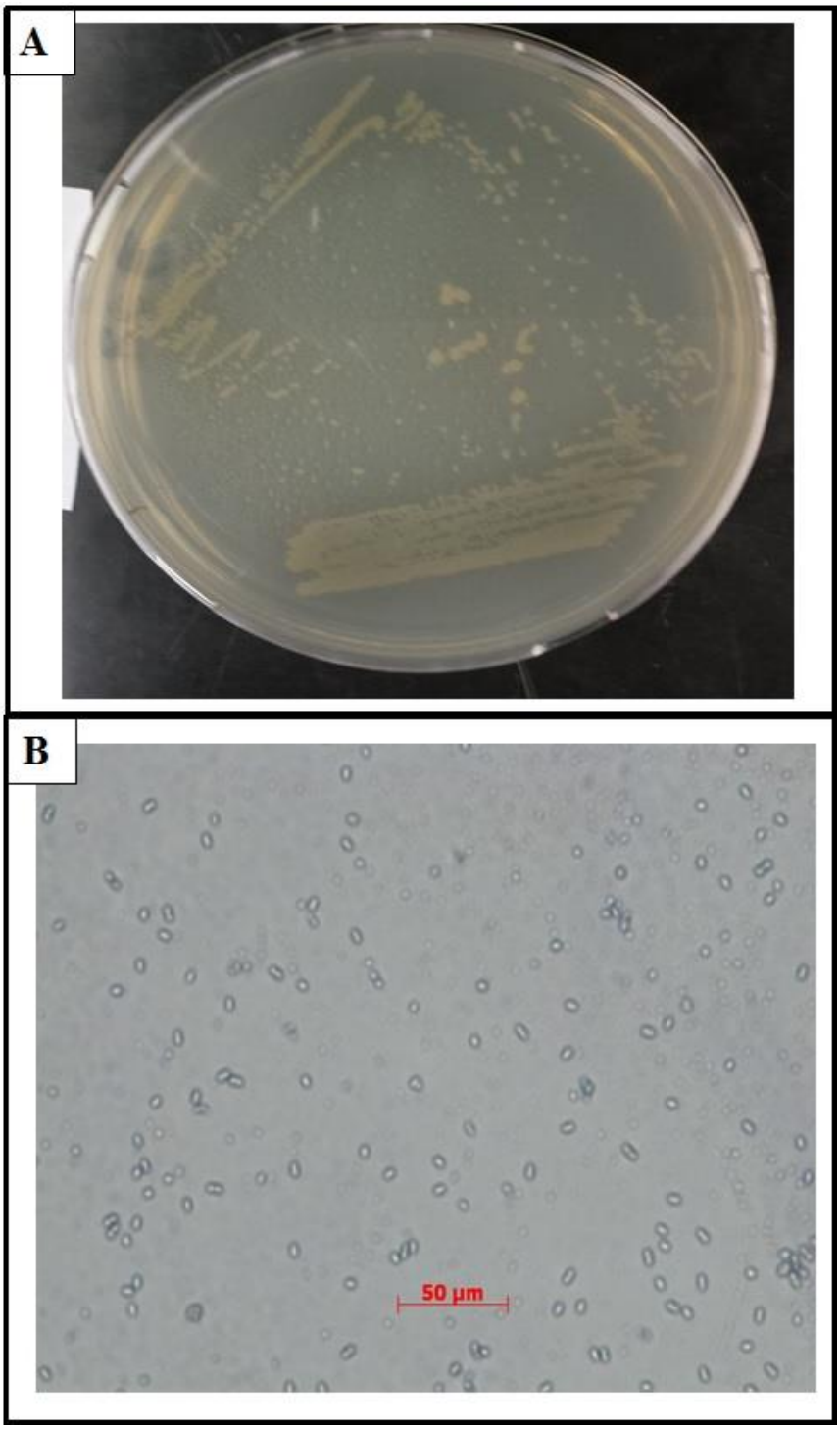


Figure 4-7. (A) Picture showing isolate BC6 colony morphology on NA plate. (B) Micrograph of isolate BC6 showing shape in a single colony (Mag. X100)

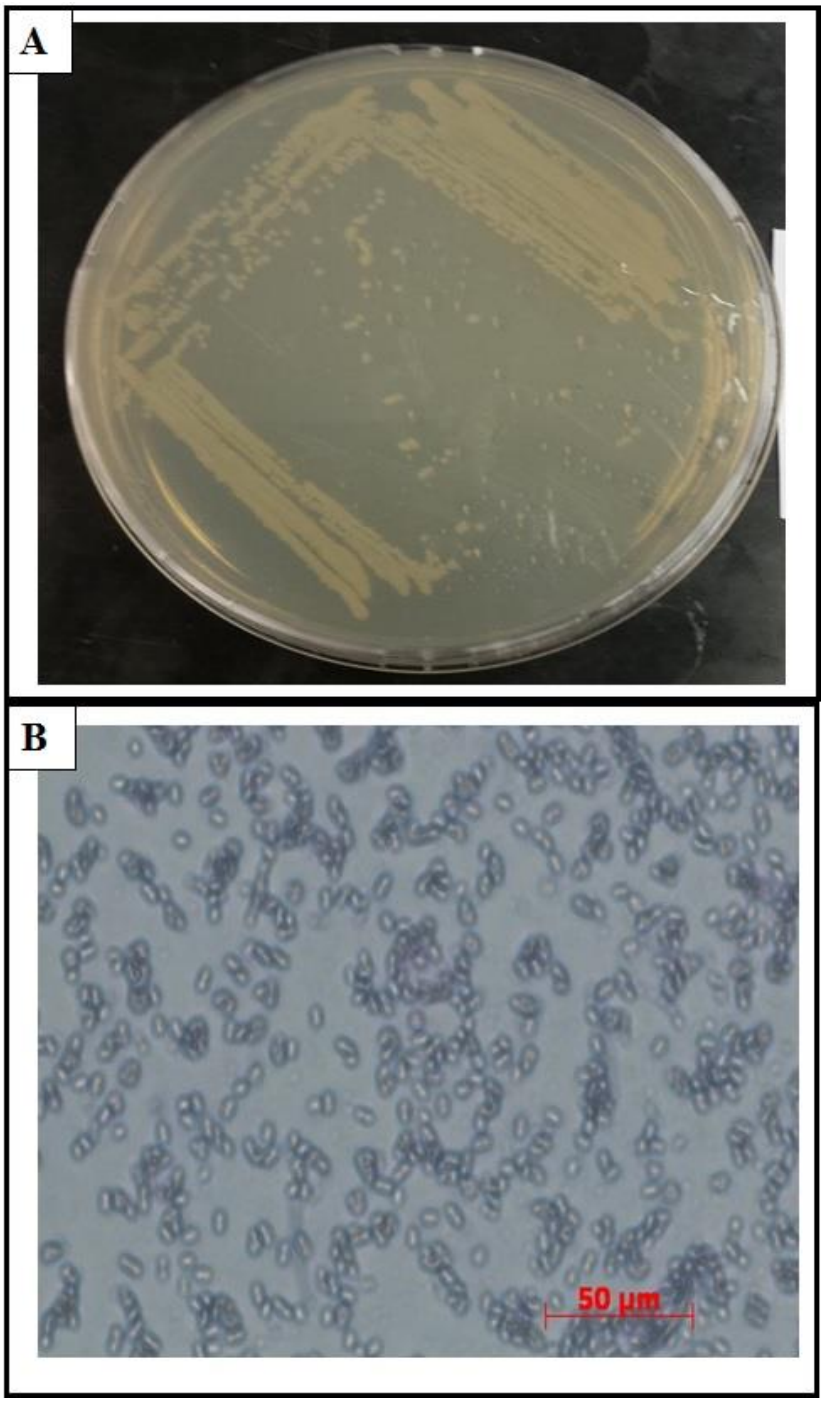


Figure 4-8. (A) Picture showing isolate BC7 colony morphology on NA plate. (B) Micrograph of isolate BC7 showing shape in a single colony (Mag. X100)

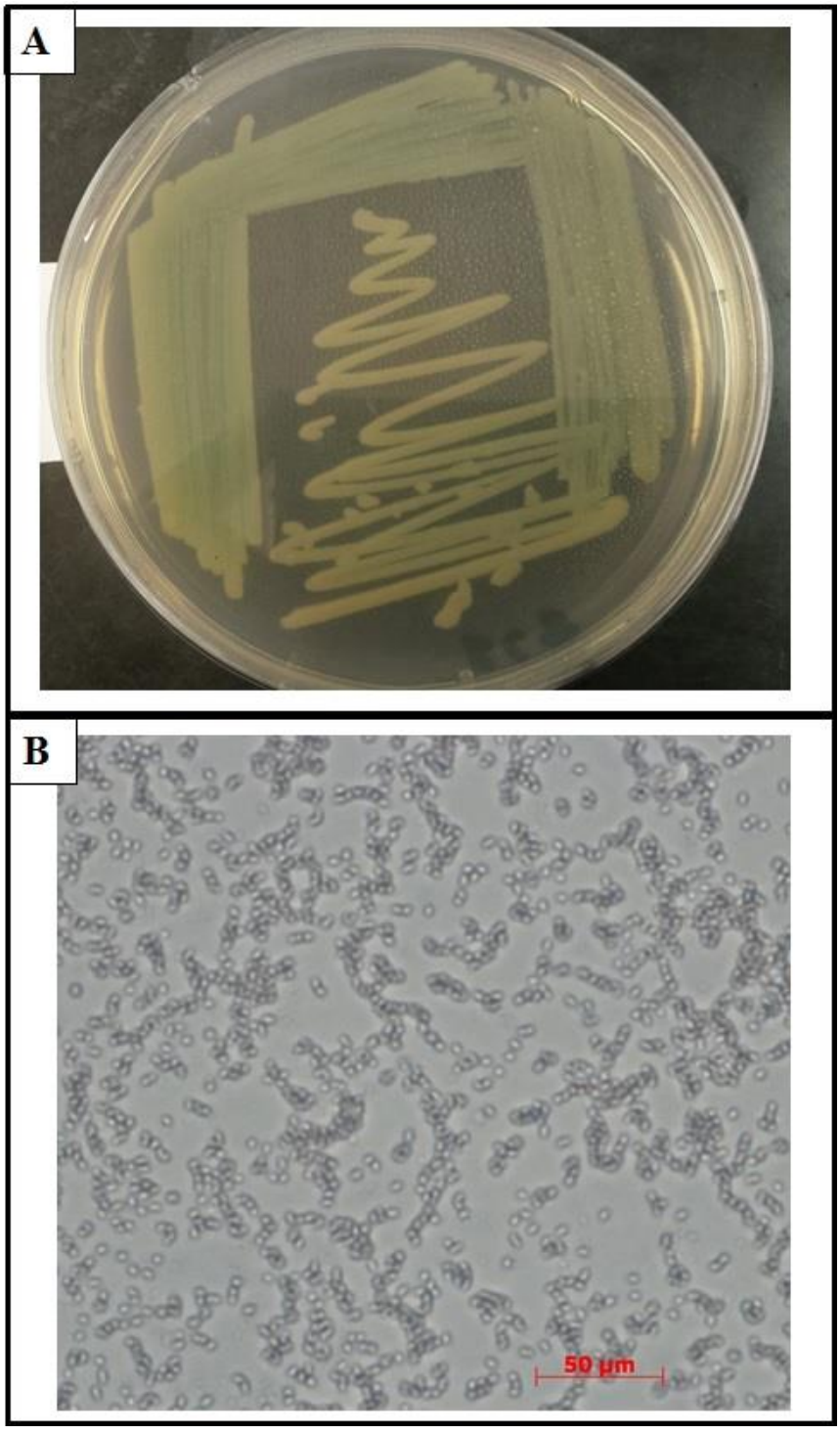


Figure 4-9. (A) Picture showing isolate BC8 colony morphology on NA plate. (B) Micrograph of isolate BC8 showing shape in a single colony (Mag. X100)

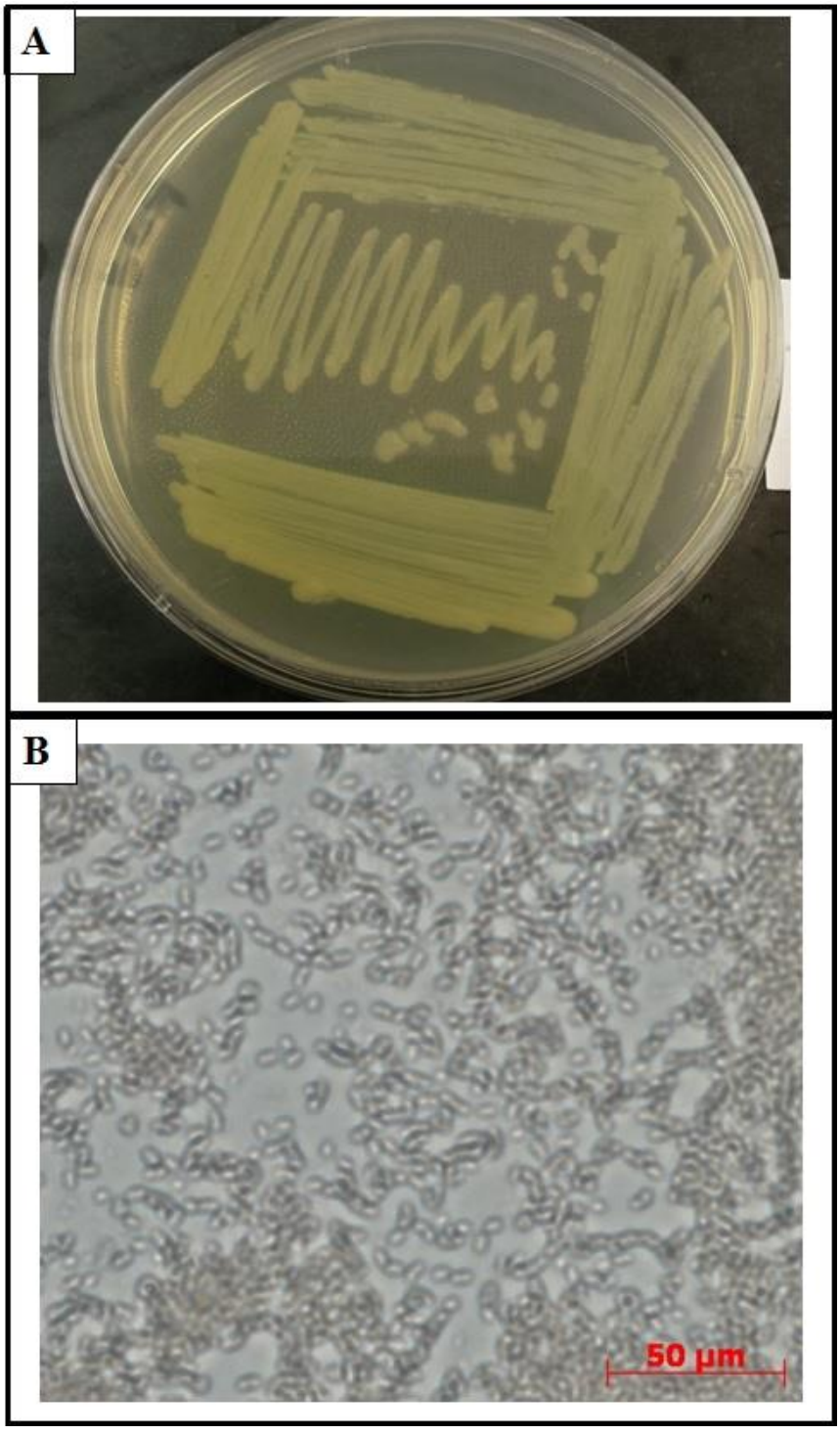


Figure 4-10. (A) Picture showing isolate BC9 colony morphology on NA plate. (B) Micrograph of isolate BC9 showing shape in a single colony (Mag. X100)

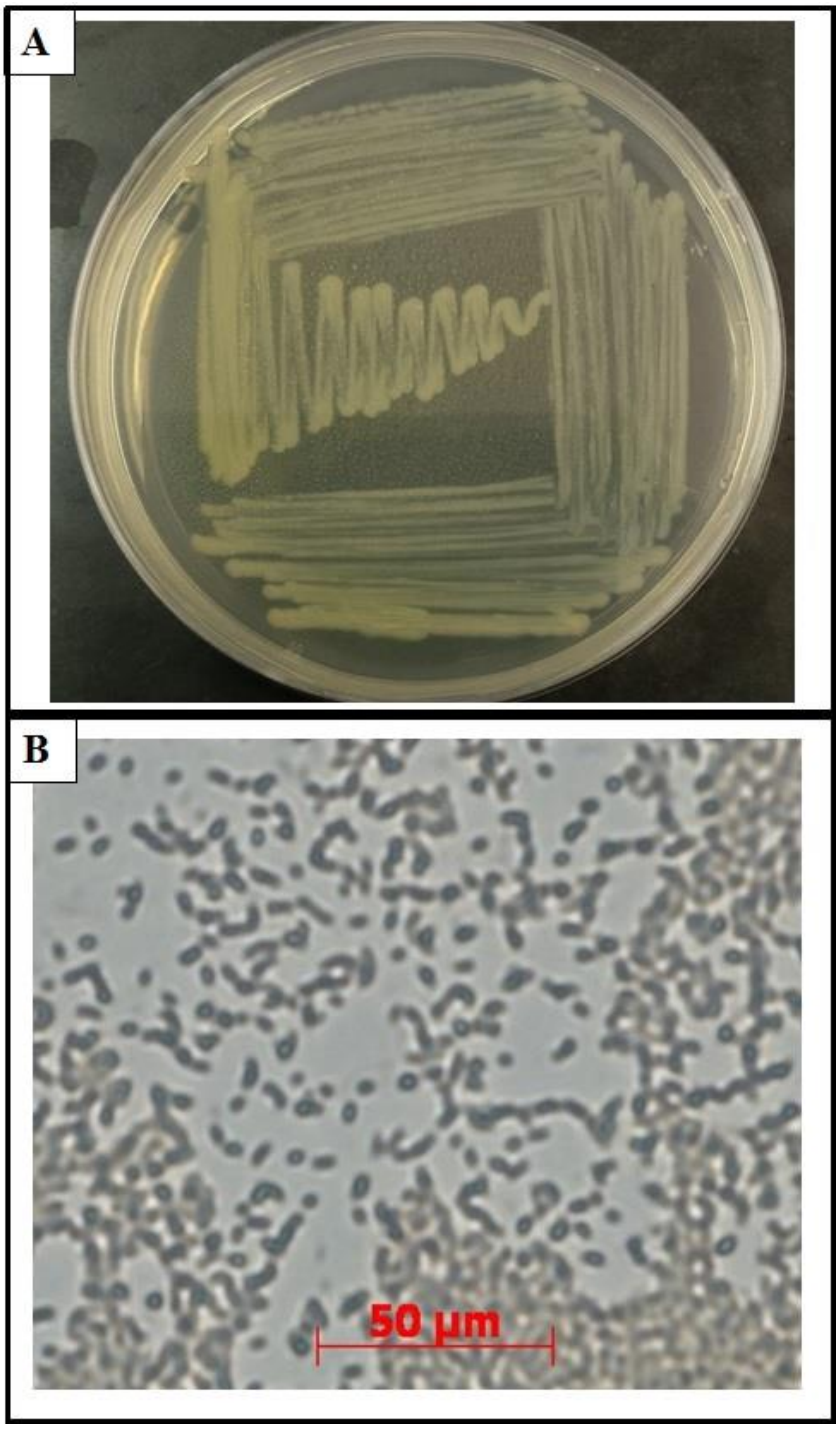


Figure 4-11. (A) Picture showing isolate BC10 colony morphology on NA plate. (B) Micrograph of isolate BC10 showing shape in a single colony (Mag. X100)

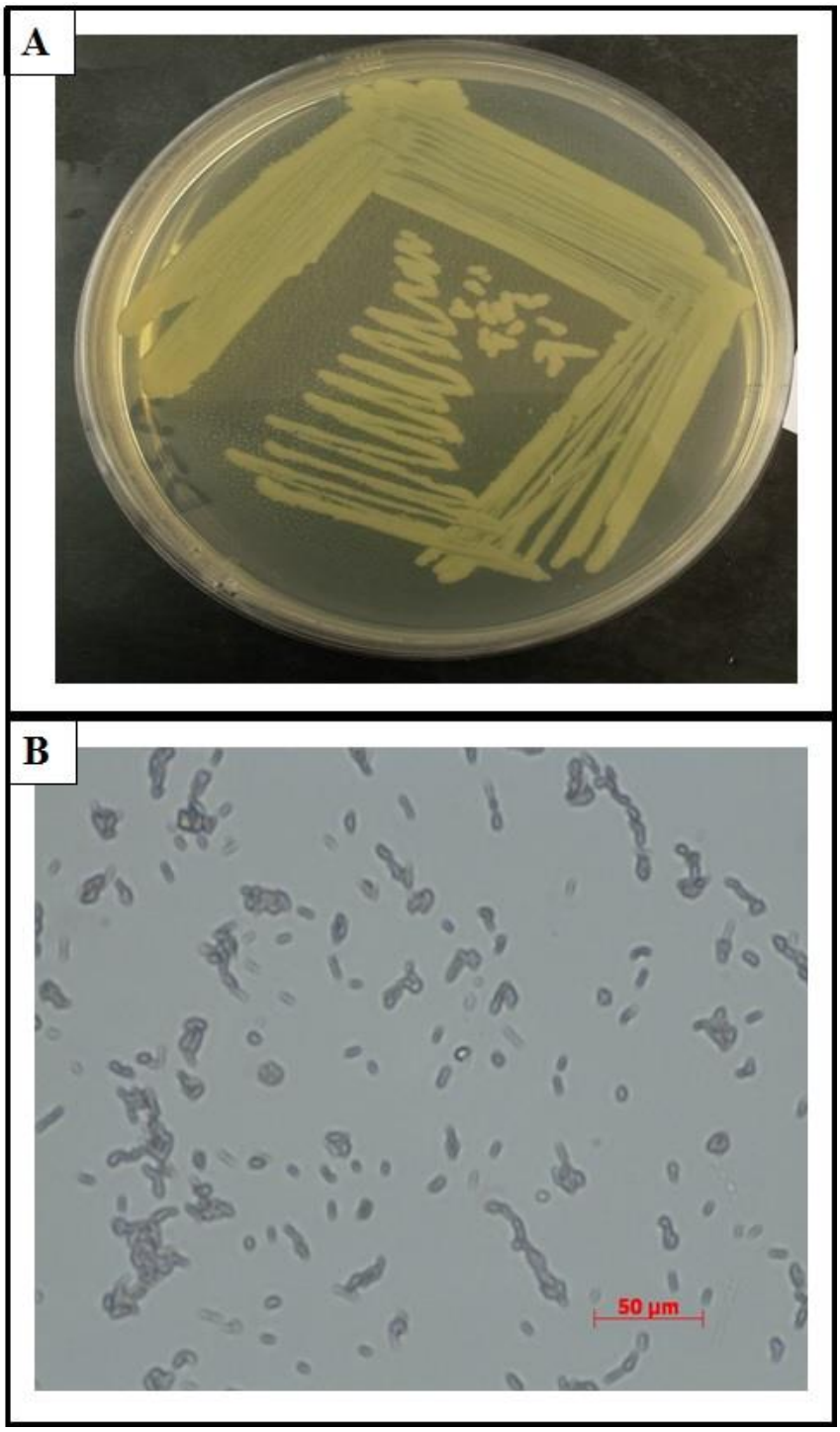


Figure 4-12. (A) Picture showing isolate DSA colony morphology on NA plate. (B) Micrograph of isolate DSA showing shape in a single colony (Mag. X100)

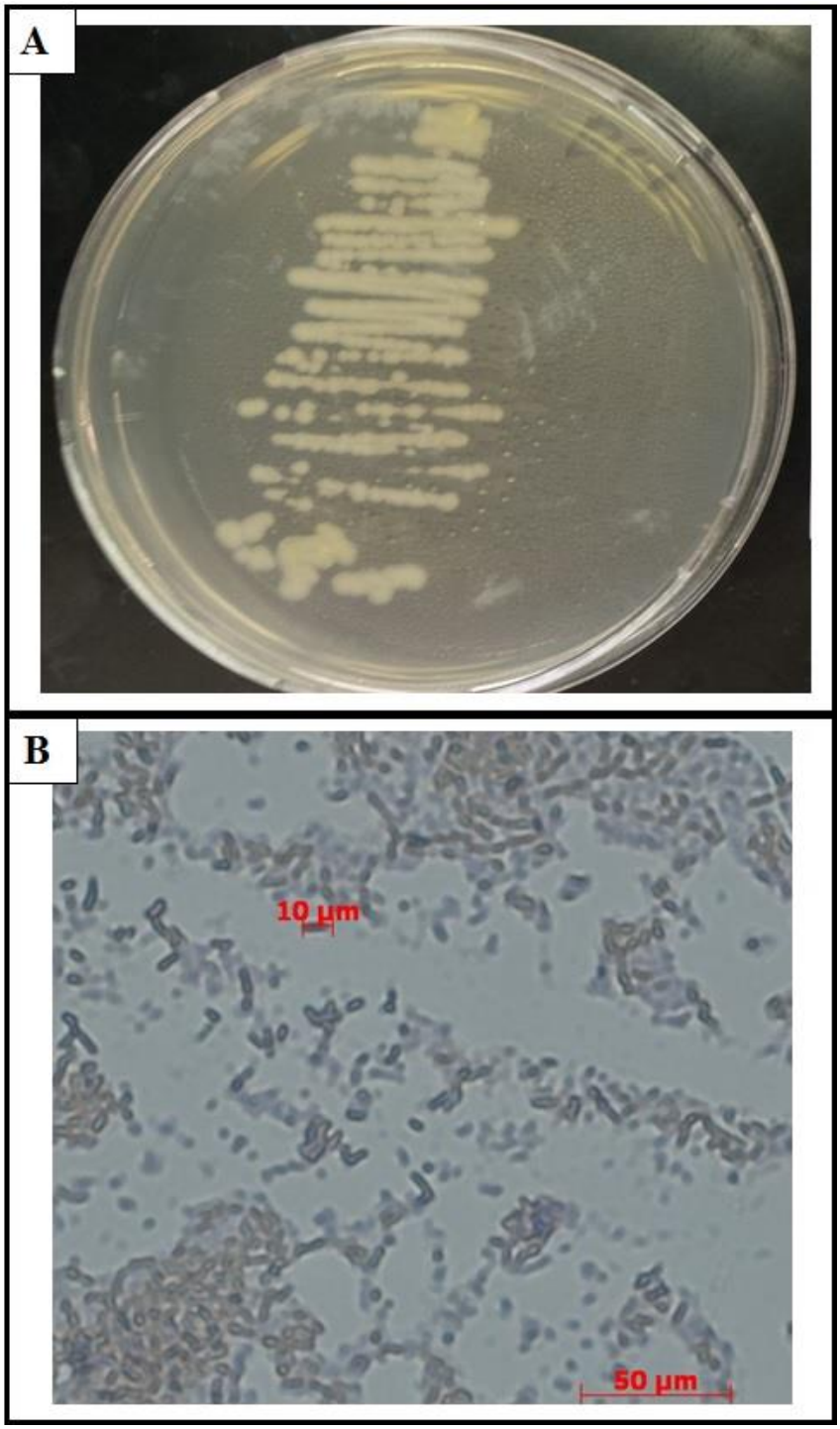


Figure 4-13. (A) Picture showing isolate DSB colony morphology on NA plate. (B) Micrograph of isolate DSB showing shape in a single colony (Mag. X100)

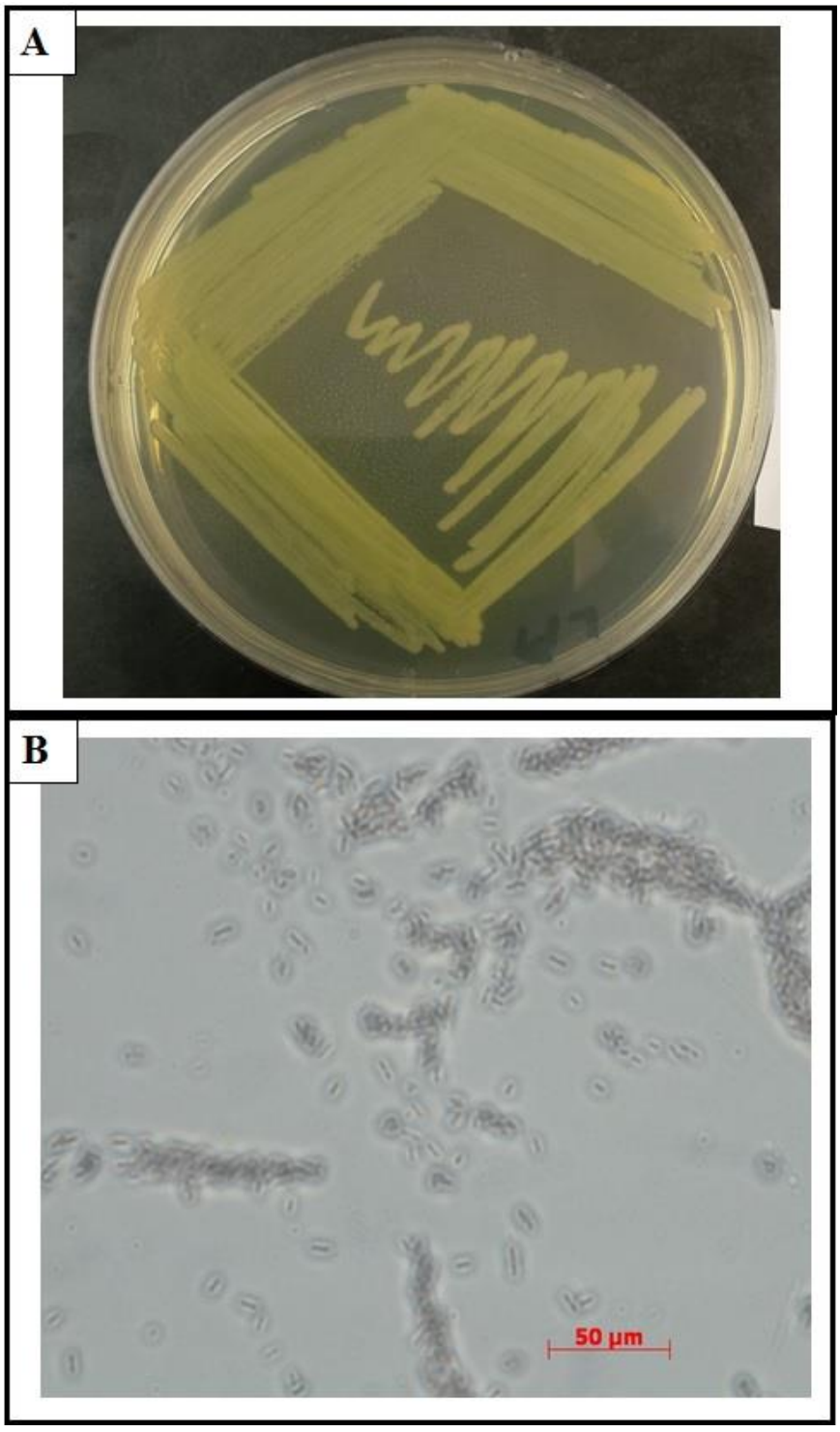


Figure 4-14. (A) Picture showing isolate LA colony morphology on NA plate. (B) Micrograph of isolate LA showing shape in a single colony (Mag. X100)

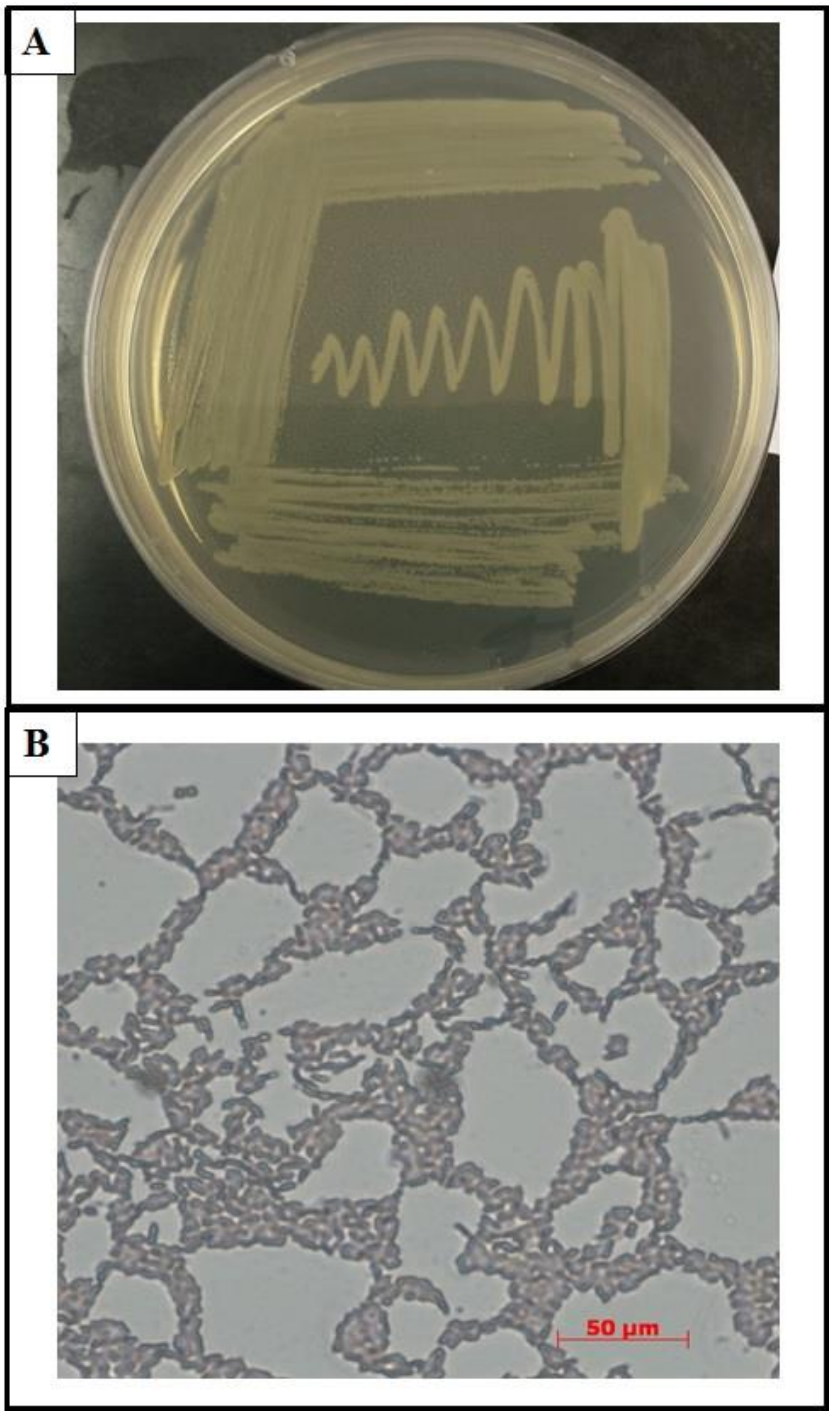


Figure 4-15. (A) Picture showing isolate LB colony morphology on NA plate. (B) Micrograph of isolate LB showing shape in a single colony (Mag. X100)

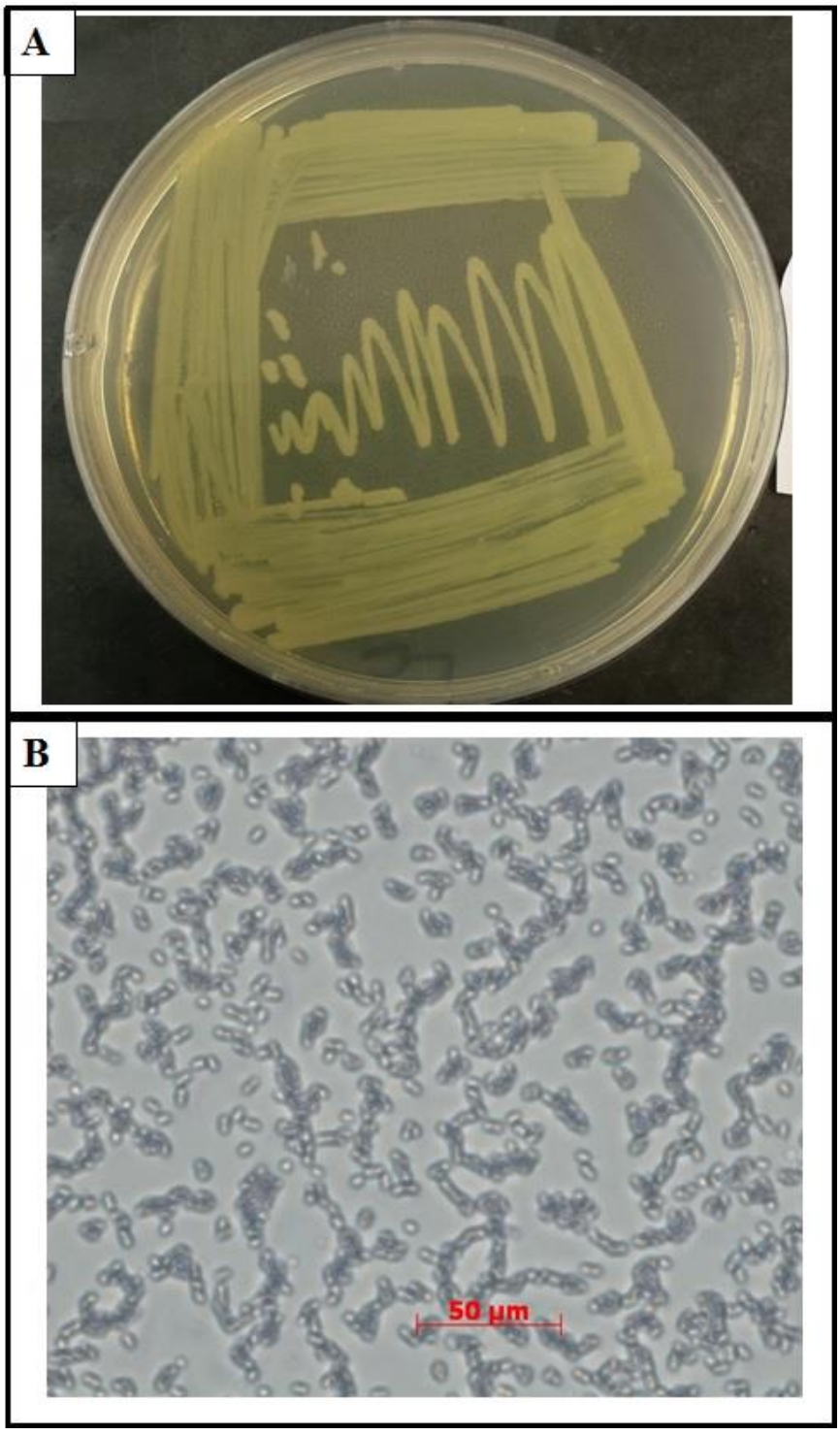


Figure 4-16. (A) Picture showing isolate LC colony morphology on NA plate. (B) Micrograph of isolate LC showing shape in a single colony (Mag. X100)

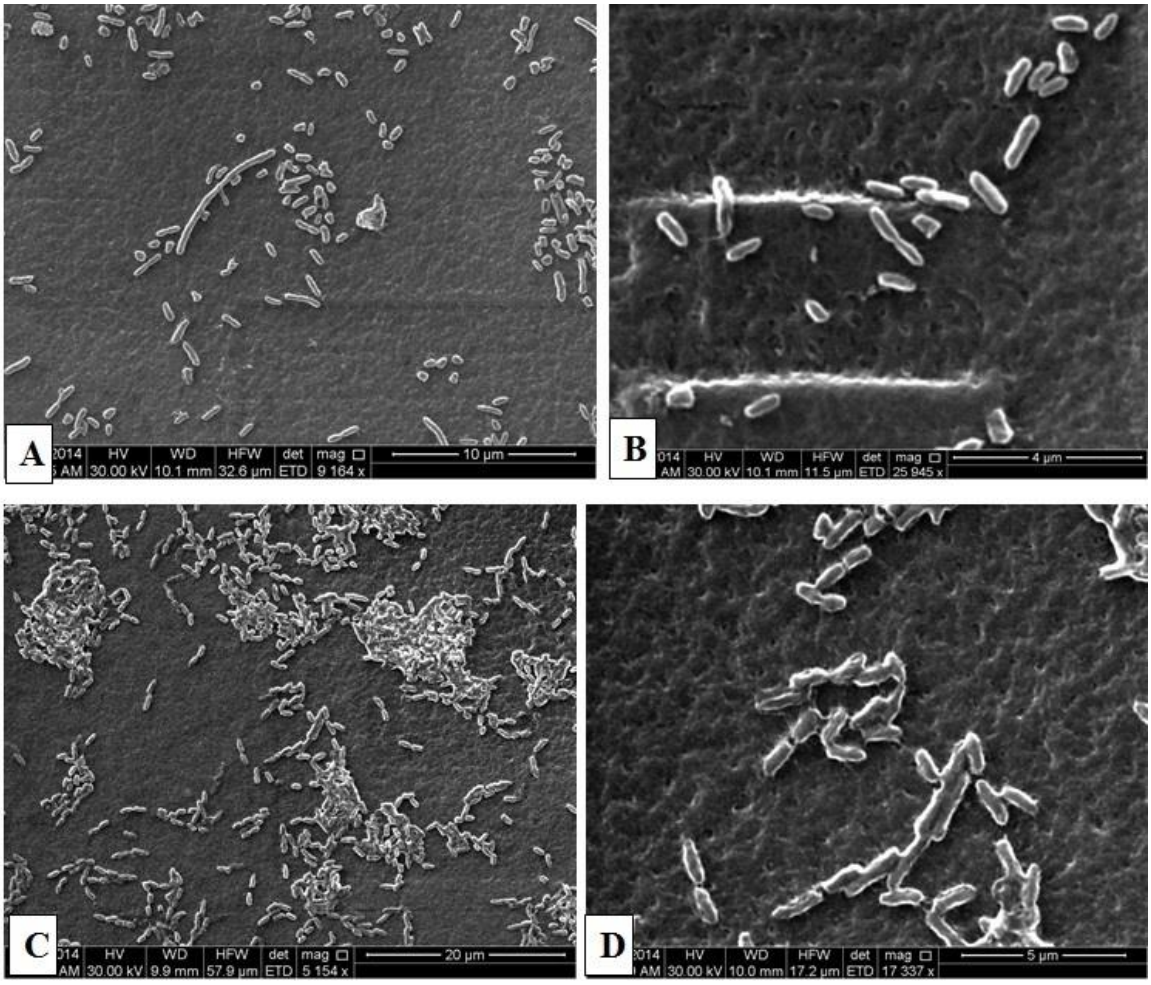


Figure 4-17. (A). SEM image of isolate BC1 (*Ochrobactrum intermedium*) at high resolution. (B). SEM image of isolate BC1 (*Ochrobactrum intermedium*) at low resolution. (C). SEM image of isolate BC6 (*Pseudomonas aeruginosa*) at high resolution. (D). SEM image of isolate BC6 (*Pseudomonas aeruginosa*) at low resolution. Magnifications of the images are shown under each of them.

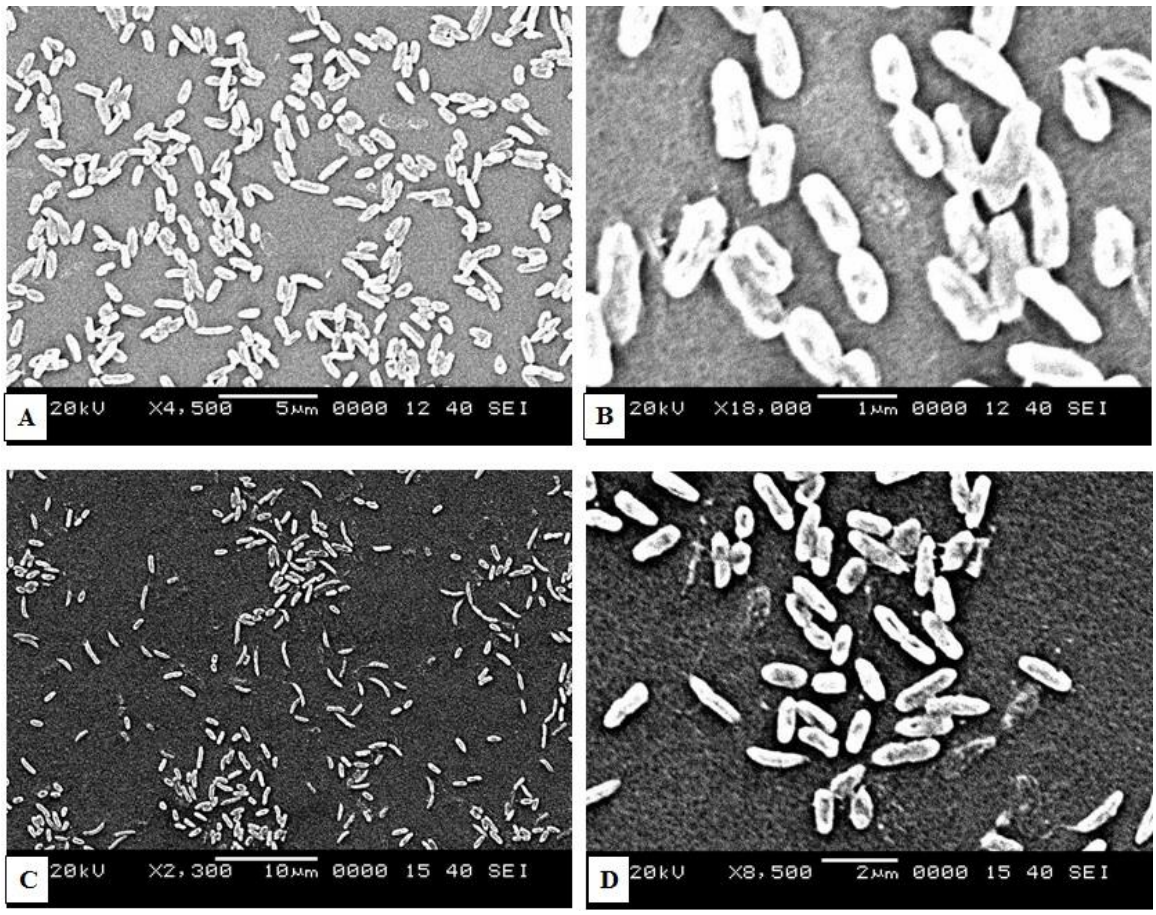


Figure 4-18. (A). SEM image of isolate BC5 (*Pseudomonas aeruginosa*) at low magnification. (B). SEM image of isolate BC5 (*P. aeruginosa*) at higher magnification. (C). SEM image of isolate BC7 (*Pseudomonas aeruginosa*) at high resolution. (D). SEM image of isolate BC7 (*P. aeruginosa*) at higher magnification. Exact magnifications of each of the images are shown under each of them.

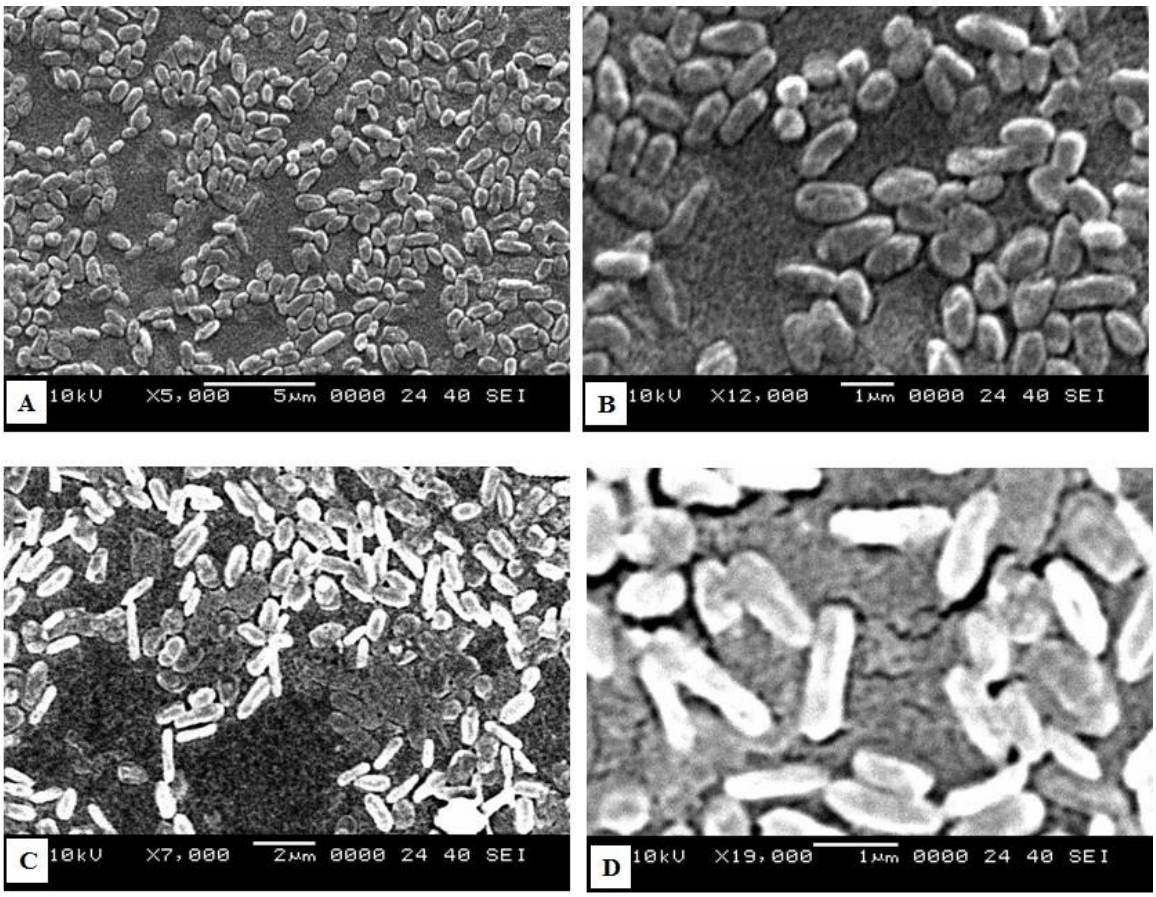


Figure 4-19. (A) SEM image of isolate DSA (*Pseudomonas aeruginosa*) at low magnification. (B). SEM image of isolate DSA (*P. aeruginosa*) at higher magnification. (C). SEM image of isolate LA (*Cupriavidus taiwanensis*) at high resolution. (D). SEM image of isolate BC7 (*C. taiwanensis*) at higher magnification. Exact magnifications of each of the images are shown under each of them.

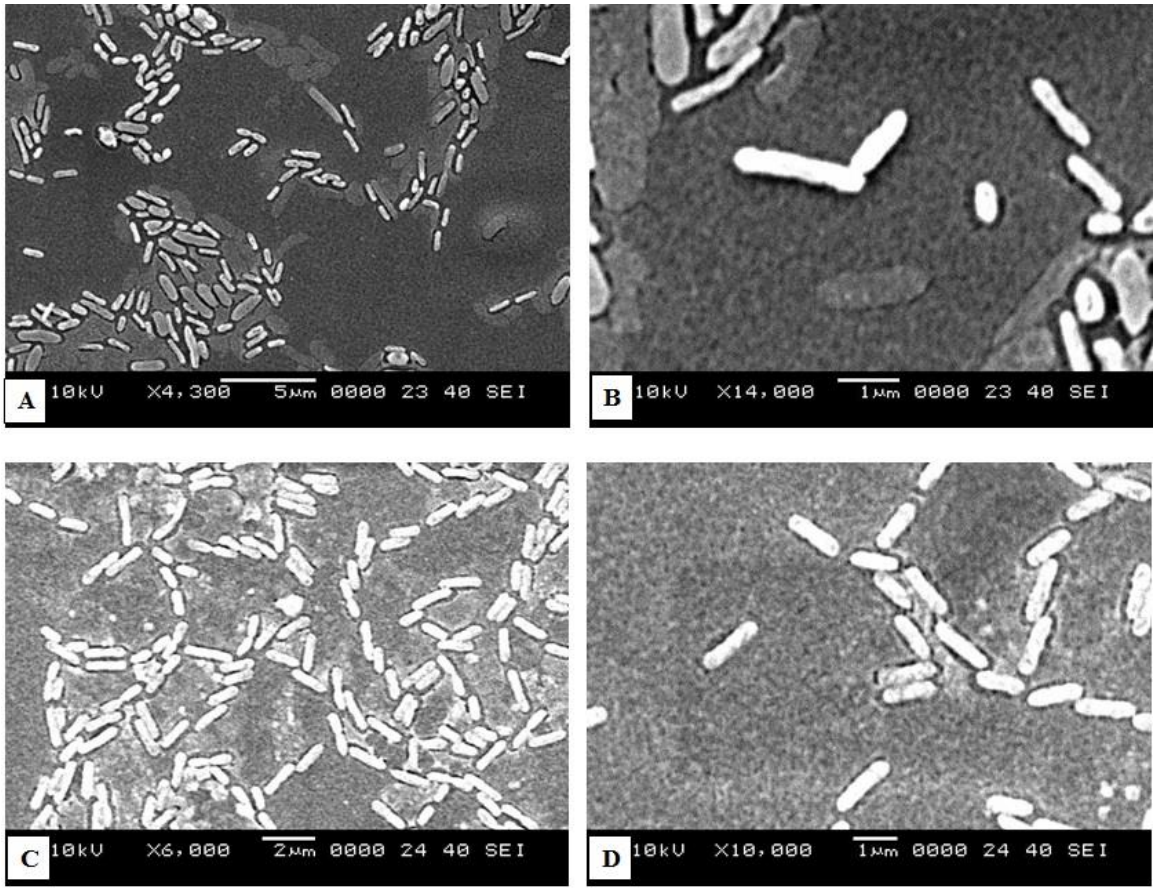


Figure 4-20. (A) SEM image of isolate LB (*Pseudomonas citronellolis*) at low magnification. (B). SEM image of isolate LB (*P. citronellolis*) at higher magnification. (C). SEM image of isolate DSB (*Pseudomonas aeruginosa*) at high resolution. (D). SEM image of isolate DSB (*P. aeruginosa*) at higher magnification. Exact magnifications of each of the images are shown under each of them.

4.3 Molecular analysis

The strains were analyzed molecularly by sequencing the conserved DNA region in all prokaryotes which is 16s rRNA. The results were used for identification of the strains and to group them phylogenetically.

4.3.1 Sequencing and identification

Isolates were identified by partial 16S rRNA gene sequences of at least 1400 base pairs (Figure 4-21, Table 4-8). The isolates belong to three distinct bacterial classes; *Alphaproteobacteria, Betaproteobacteria* and *Gammaproteobacteria* as shown in (Figure 4-22). Members of the *Class Alphaproteobacteria* are the majority constituting about 87% (13 out of 15) of all isolates. Of the 13 γ-proteobacteria isolates, only one (i.e isolate JBL_LB) is a strain of the species *Pseudomonas citronellolis*, the remaining are strains of *P. aeruginosa* (Table 4-7). Isolate JBL_BC1 is a member of Class α-proteobacteria and belongs to family Brucellaceae. It shares close identity to *Brucella sp.* and *Ochrobactrum sp*. Isolate in the β-proteobacteria class is JBL_LA. It is in the family Burkholderiaceae and its closest relative according to BLAST results is *Cupriavidus taiwanensis*. All the isolates have more than 98% identity to their relatives.

Table 4-7. Isolate characteristics based on 16S rRNA sequencing

Strain label	Closest match	Identity	Class	Accession no.
JBL_BC1	*Ochrobactrum sp.*	99%	α-proteobacteria	KP792293
JBL_BC2	*Pseudomonas aeruginosa*	100%	γ-proteobacteria	KP662550
JBL_BC3	*P. aeruginosa*	100%	γ-proteobacteria	KP662548
JBL_BC4	*P. aeruginosa*	99%	γ-proteobacteria	KP662547
JBL_BC5	*P. aeruginosa*	99%	γ-proteobacteria	KP792289
JBL_BC6	*P. aeruginosa*	100%	γ-proteobacteria	KP662549
JBL_BC7	*P. aeruginosa*	99%	γ-proteobacteria	KP792290
JBL_BC8	*P. aeruginosa*	99%	γ-proteobacteria	KP792291
JBL_BC9	*P. aeruginosa*	100%	γ-proteobacteria	KP662551
JBL_BC10	*P. aeruginosa*	100%	γ-proteobacteria	KP792292
JBLDSA	*P. aeruginosa*	99%	γ-proteobacteria	KP683357
JBL_DSB	*P. aeruginosa*	99%	γ-proteobacteria	KP792288
JBL_LA	*Cupriavidus taiwanensis*	98%	β-proteobacteria	KP792294
JBL_LB	*P. citronellolis*	99%	γ-proteobacteria	KP792286
JBL_LC	*P. aeruginosa*	100%	γ-proteobacteria	KP792287

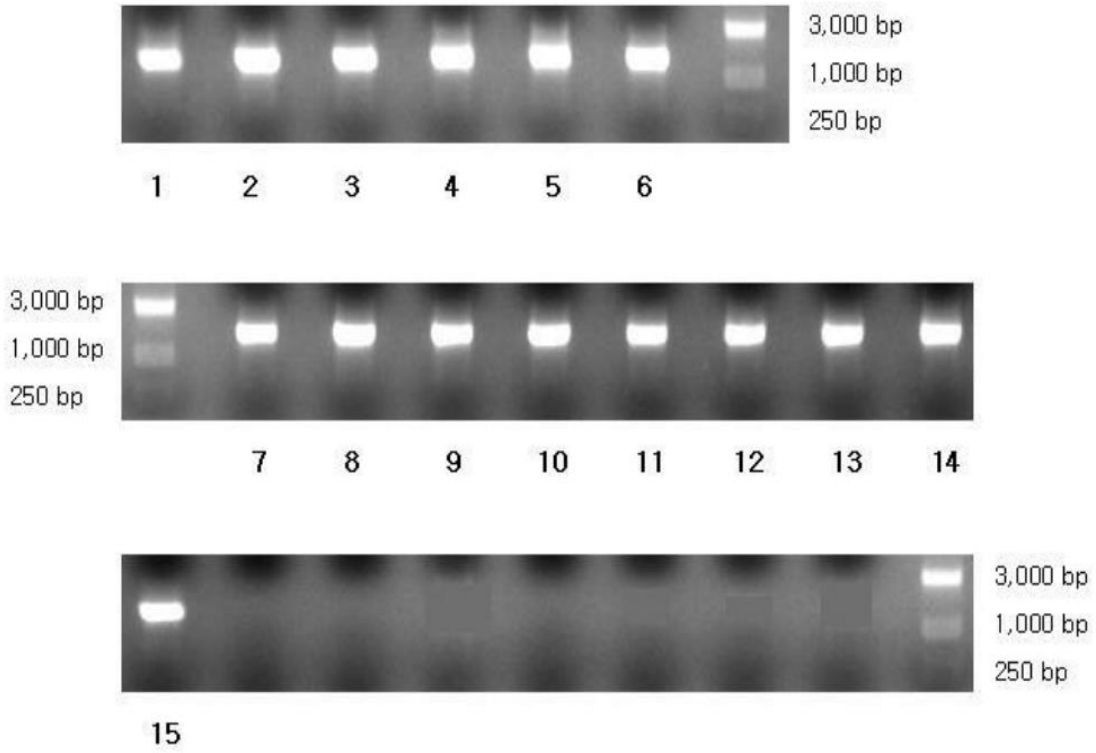


Figure 4-21. Electrophoresis gel image for all the 15 isolates to determine their DNA length (*see Table 4-8 for legend*).

Table 4-8. Gel image legend showing size of DNA base pairs used in the 16S rDNA analysis and G+C content

Number	Strain label	Size of DNA (base pairs)	G+C %
1	LA	1506 bp	52.72
2	LB	1694 bp	54.84
3	LC	1695 bp	55.63
4	DSA	1661 bp	52.44
5	DSB	1356 bp	52.88
6	BC1	1659 bp	55.64
7	BC2	1693 bp	54.64
8	BC3	1697 bp	54.68
9	BC4	1679 bp	54.73
10	BC5	1699 bp	54.56
11	BC6	1695 bp	54.63
12	BC7	1702 bp	54.58
13	BC8	1704 bp	54.58
14	BC9	1703 bp	54.43
15	BC10	1697 bp	54.51

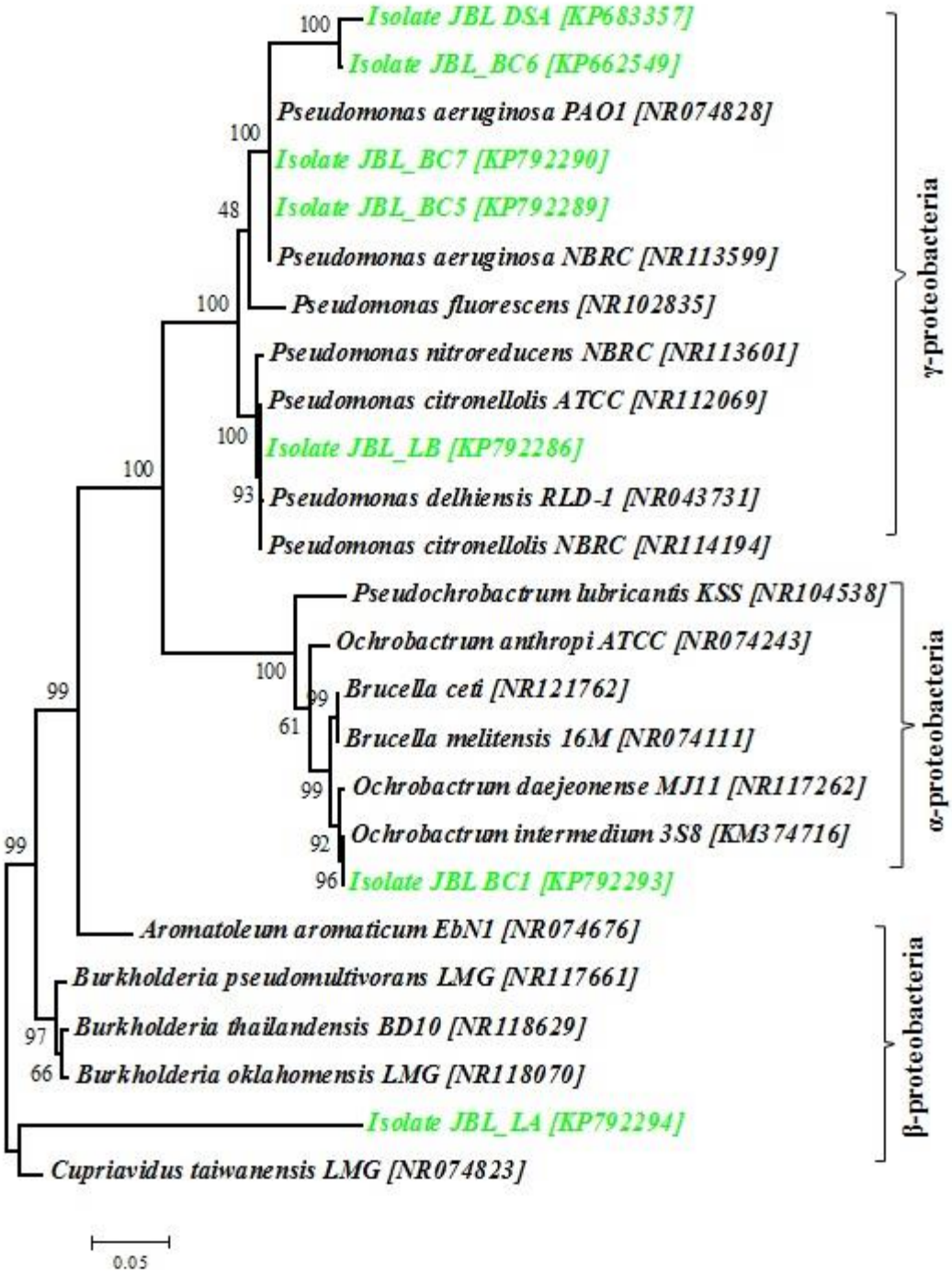


Figure 4-22. Phylogenetic tree of the important isolates showing their closest relatives and likely ancestors. Accession numbers of each strains are written in parenthesis.

4.3.2 Phylogenetic grouping

Phylogenetic trees were constructed to estimate evolutionary history of the strains by Neighbor-joining method. Figure 4-23 reveals that all the isolates are closely related to the members of their suspected family and genera except isolate JBL_LA. Although the isolate shares branch with *Cupriavidus sp.* and *Ralstonia sp.*, its very long branch length and low bootstrap value (53%) shows that it is clearly an outgroup just like how *Bifidobactrum bifidum* (bootstrap value, 48%) is an outgroup to members of *Brucellae* family (see Figure 4-23).

The long evolutionary distances of these outgroups to other strains make the analysis of relationships between other closer strains clumsy on the same tree. A separate tree was therefore constructed for the remaining closely related strains sharing shorter roots. Figure 4-24 shows that isolate JBL_BC1 has a close relationship with *Brucella melitensis* as well as *Ochrobactrum intermedium*. While the former is a renown pathogen (He, 2012) in human and livestocks, the latter has a proven record in PAH degradation especially, high molecular weight (HMW) PAH such as pyrene and benzo(a)pyrene (Luo et al., 2009). From the tree, BC1 is closer to *O. intermedium* than *B. melitensis.*

From the phylogenetic clusters of genus *Pseudomonas*, isolate JBL_LB is definitely a strain of *P. citronellolis*, while other isolates are that of *P. aeruginosa*. Species of this (Ghosh et al., 2014; Pasumarthi et al., 2013) genus are widespread bacteria and *P. aeruginosa* is particularly an established hydrocarbon degrader. Unfortunately, it has also been confirmed to be an opportunistic human pathogen (Stover et al., 2000). On the other hand, *P. citronellolis*, a degrader of citronellol (3,7-dimethyl-6-octen-1-ol) and a bio-

surfactant producer (Jacques et al., 2008) has not been implicated as a pathogen. However, there is almost no record of its use in PAH-biodegradation. This isolate was therefore selected with others for biodegradation experiments.

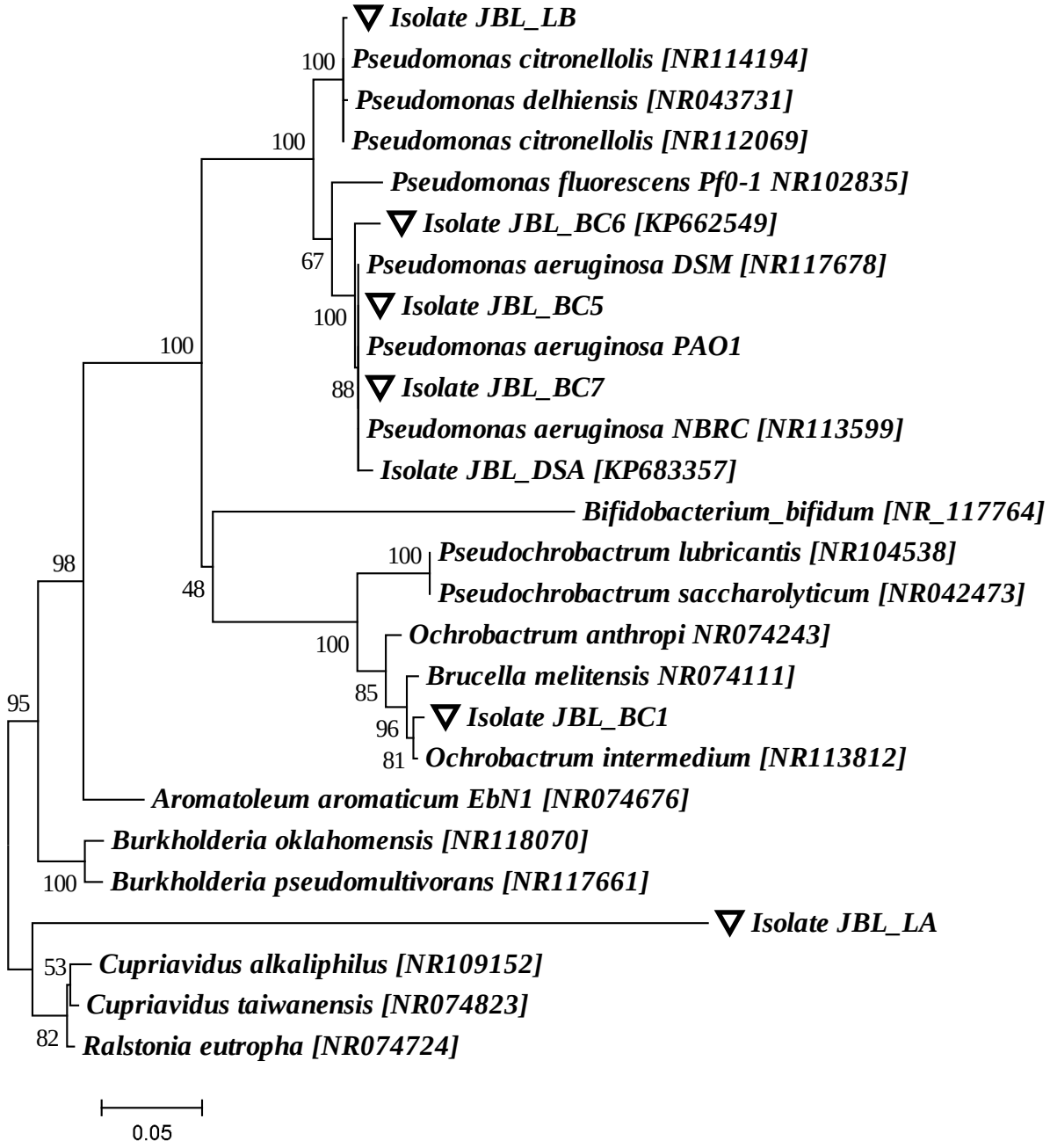


Figure 4-23. Phylogenetic tree of the isolates showing Isolate JBL_LA as an Outgroup. The tree branch lengths are drawn to scale and are equal to the evolutionary distances (Kimura, 1980) for estimation of phylogeny. Bootstrap values shown are the nodes are in percent. The scale 0.05 represents sequence divergence.

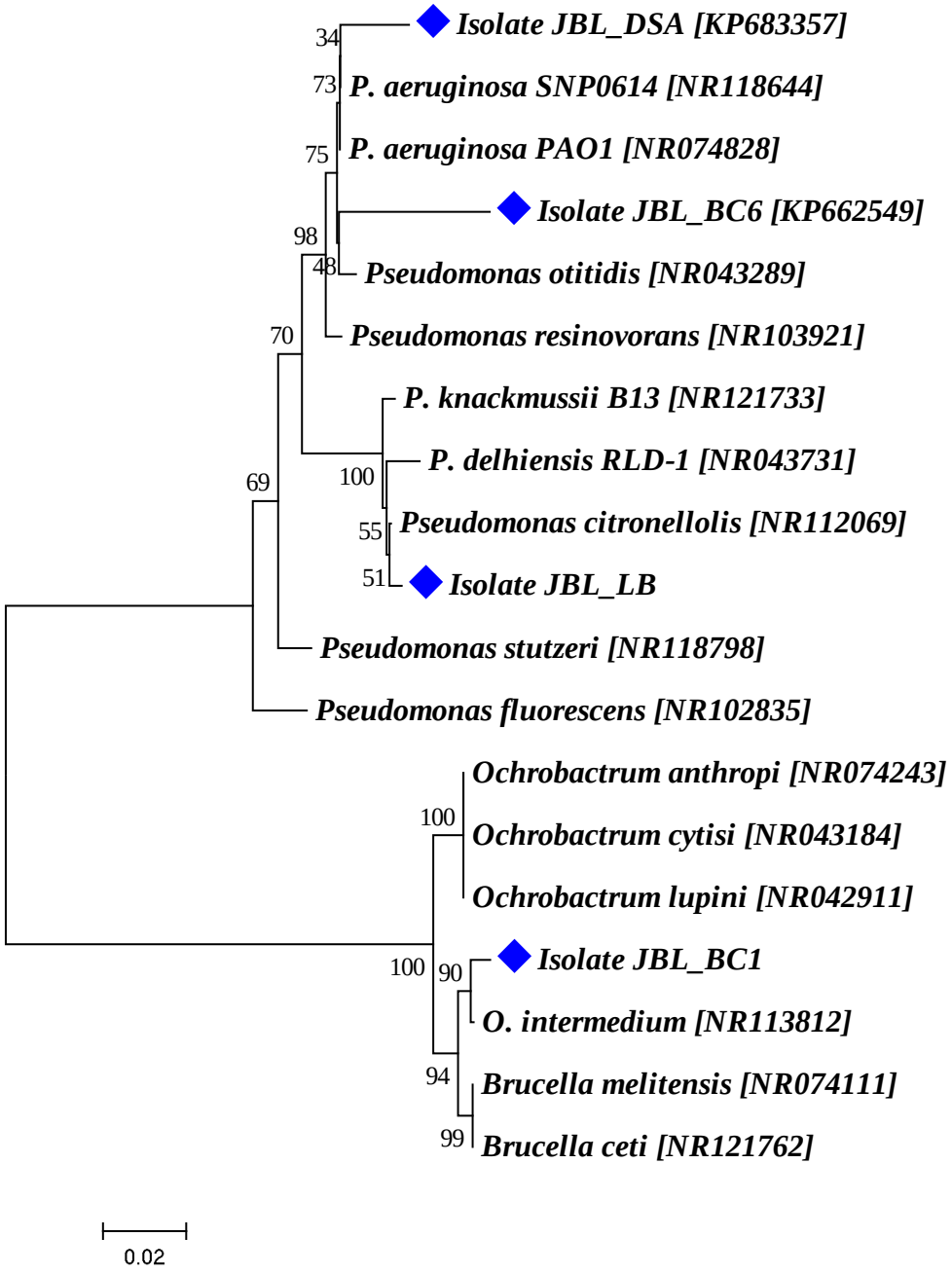


Figure 4-24. Phylogenetic tree of *Pseudomonaceae* and *Brucellaceae*. The scale of sequence divergence is 2% as shown. Isolates of interest are marked with big blue dot-mark. BC2 to BC10 are very closely related strains, hence only BC6 is included in this tree.

4.4 Degradation results

Molecular analyses revealed that the isolates were basically strains of four distinct species. Isolates in each of the four distinct species were tested for their abilities to degrade PAH and diesel. Since *P. aeruginosa* is arguably one of the best hydrocarbon-degraders, 3 strains (isolates BC5, BC6 and BC7, observed as best hydrocarbon-utilizers Table 4-4) were therefore compared with each other. While other isolates that are strains of other species (i.e BC1, LA and LA) were also compared with each other.

4.4.1 Degradation of phenanthrene

The degradation ability of the three strains, BC5, BC6 and BC7, were assessed in a 100-ml BH mineral broth containing 100 mg/L of phenanthrene as sole carbon source. The strains utilized the carbon source, and in 15 days, their degradation of phenanthrene was found to be from 63% up to as much as 96% (Figure 4-25). Isolate BC5 was found to be most efficient in degradation reaching 95% degradation within 12 days. BC7 has the least degradation achieving less than 70% reduction in phenanthrene concentration within the experimental period. A similar pattern of degradation rate is observed in all the 3 strains. The rate starts smoothly until day 9, it then proceeds exponentially till day 12 and finally slows down rapidly.

Similar experiments to test phenanthrene-degradation ability of isolates LA, LB and BC1 indicated that they are equally better isolates. For instance, isolate LB (*P. citronellolis*) has phenanthrene-degrading ability (above 94%) that can compete favorably with isolate BC5

which is a strain of well-known PAH-degrader, *P. aeruginosa* (Figure 4-26). Isolates LA (close relative of *Cupriavidus taiwanensis*) and BC1 (*Ochrobactrum intermedium*), also performed better than BC6 and BC7.

All the isolates have similar growth pattern in phenanthrene-containing culture (Figure 4-27) and there is a direct relationship between bacterial population and amount (in percent) of PAH degraded (Figure 4-28). Bacterial growths assessment by optical density measurement gives no relationship with bacterial population assessed by counting colony-forming units.

Representative chromatograms of the phenanthrene degradation profiles (shown in Figure 4-29) indicate a substantial decrease in the concentrations of the phenanthrene in the culture over time (in days).

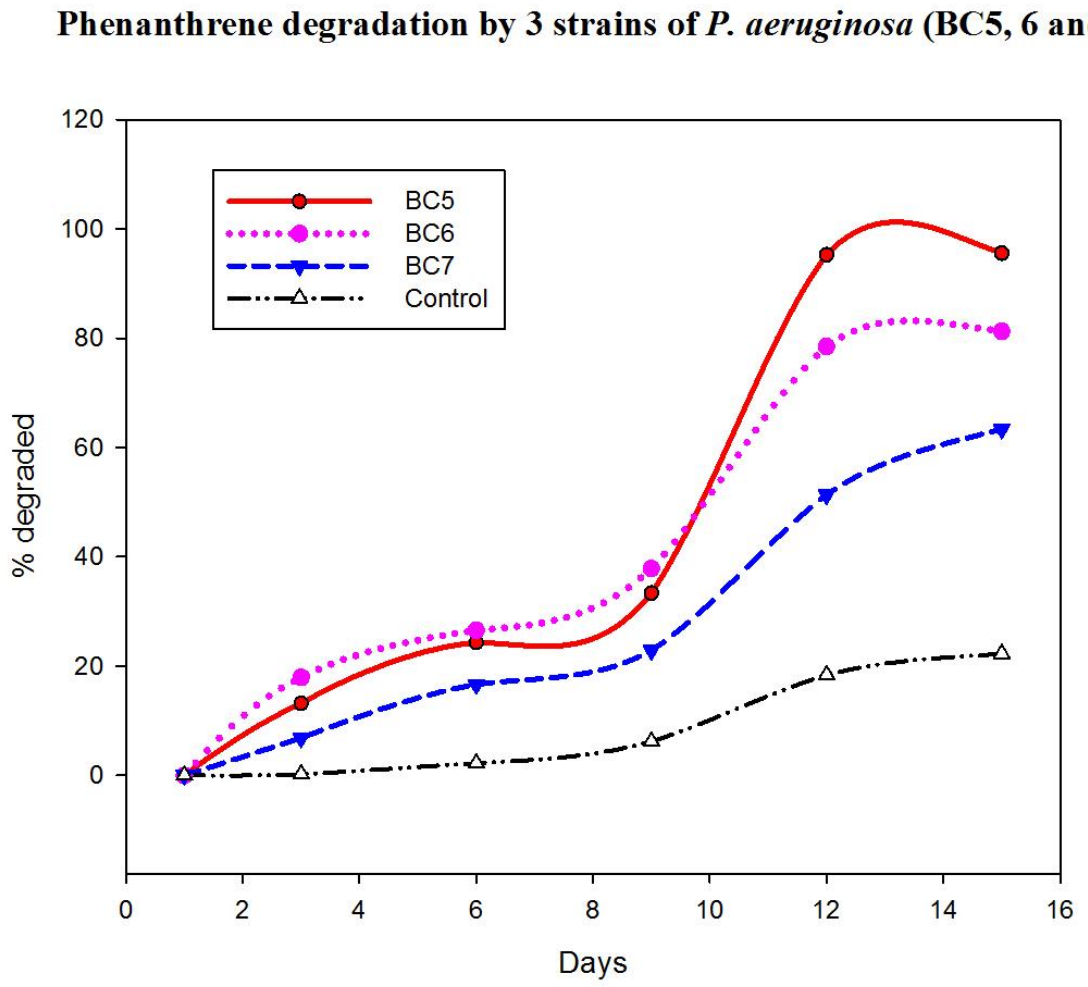


Figure 4-25. Biodegradation of phenanthrene by three different strains (BC5, 6 and 7)

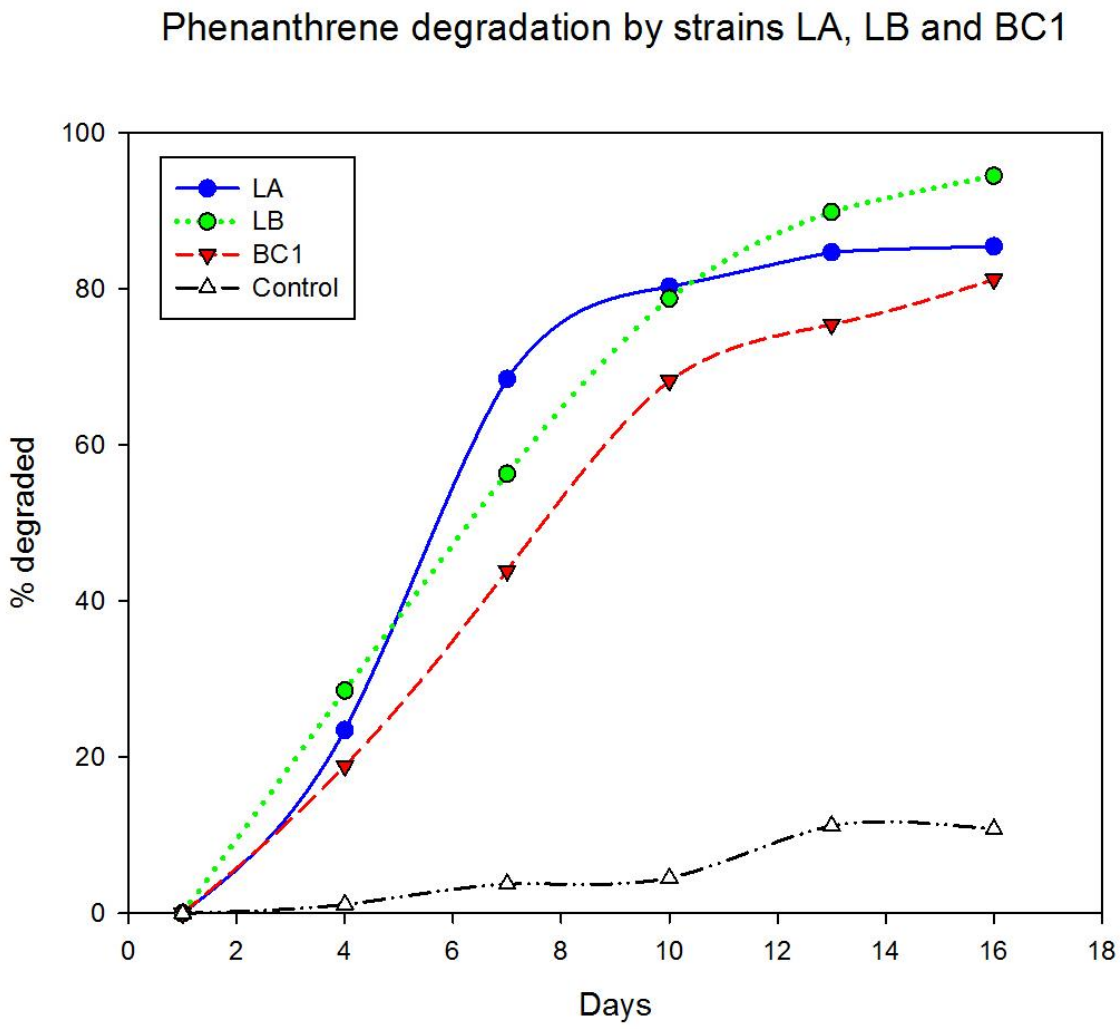


Figure 4-26. Biodegradation of phenanthrene by isolates LA, LB and BC1

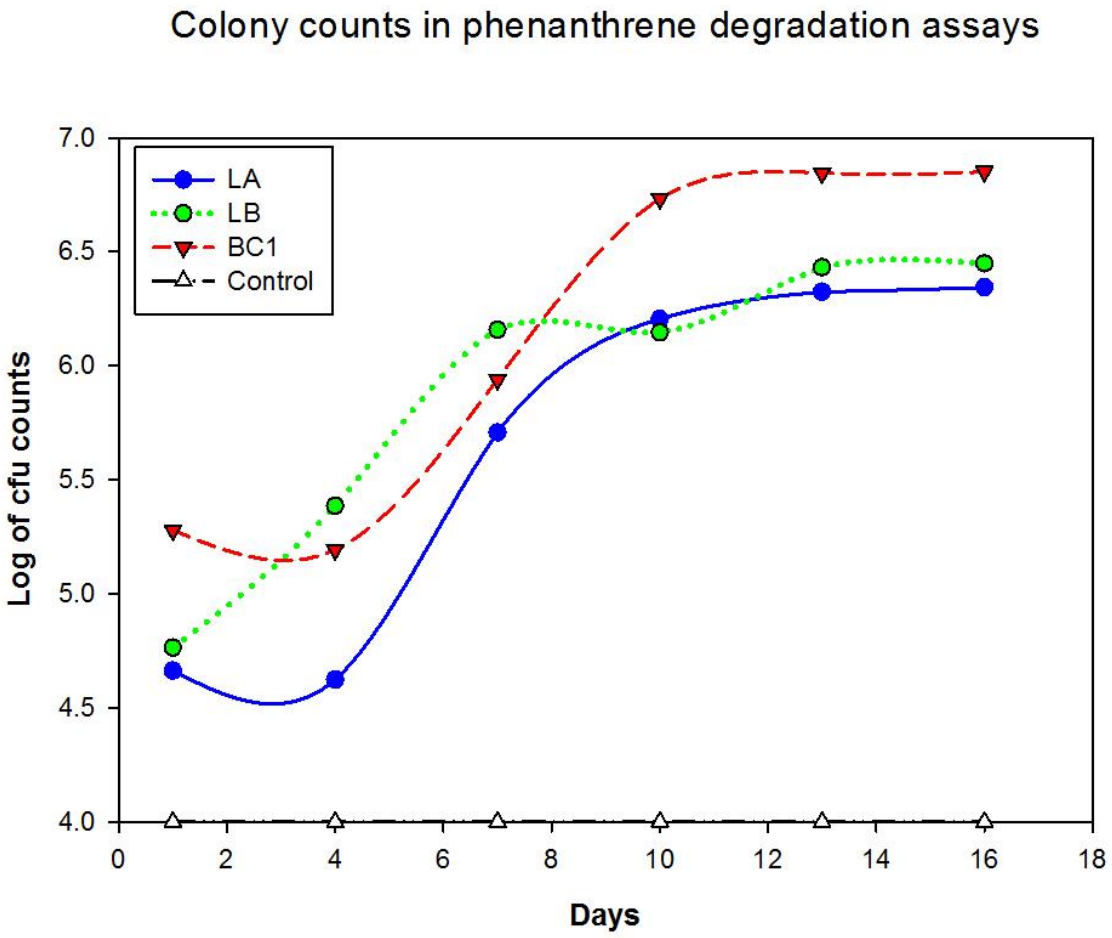


Figure 4-27. Growth curves of isolates LA, LB and BC1. Growth is estimated as logarithm of number of colony forming unit per ml (cfu/ml) of degradation culture

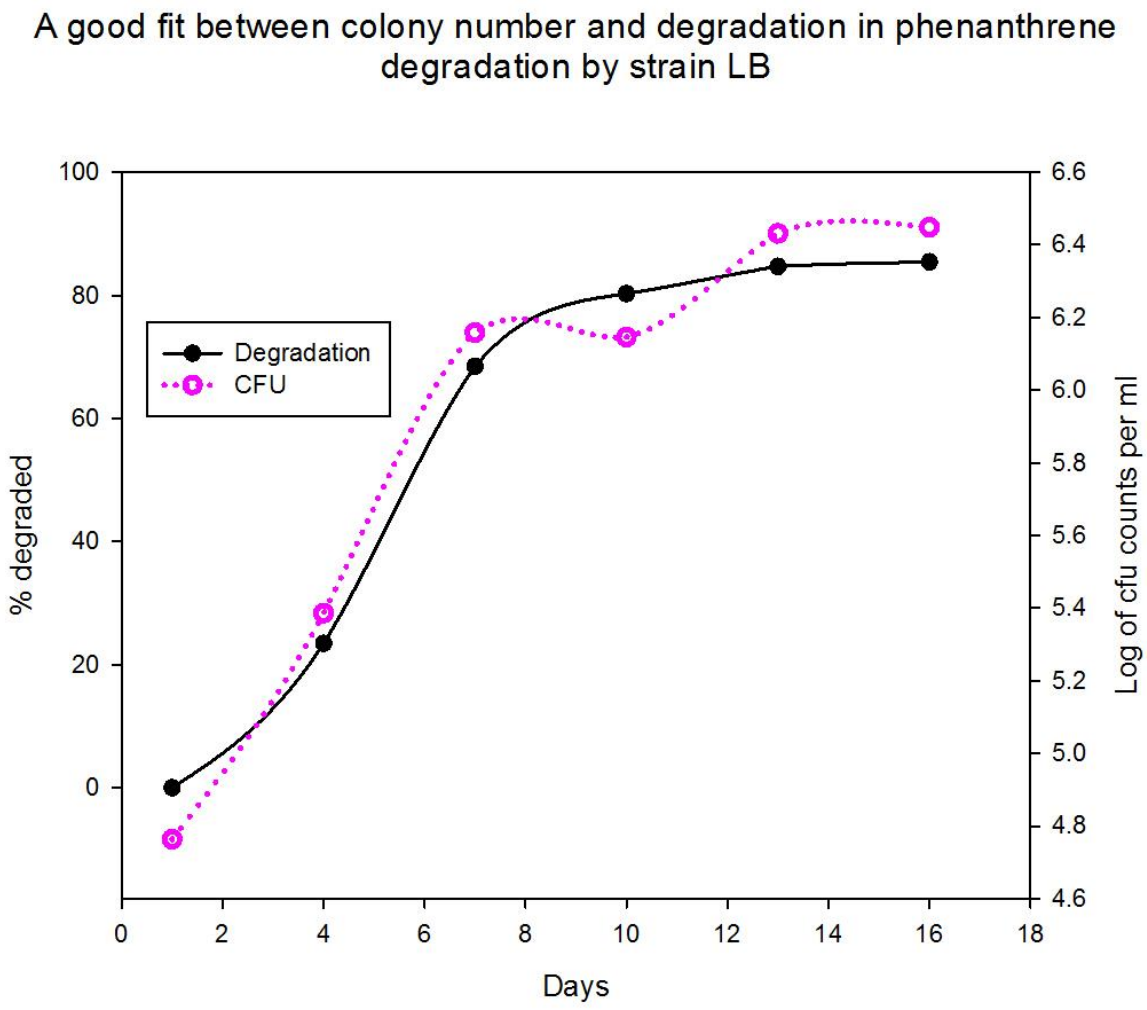


Figure 4-28. Degradation and bacterial population has a direct relationship. Population is estimated by counting number of colony forming units per ml of culture.

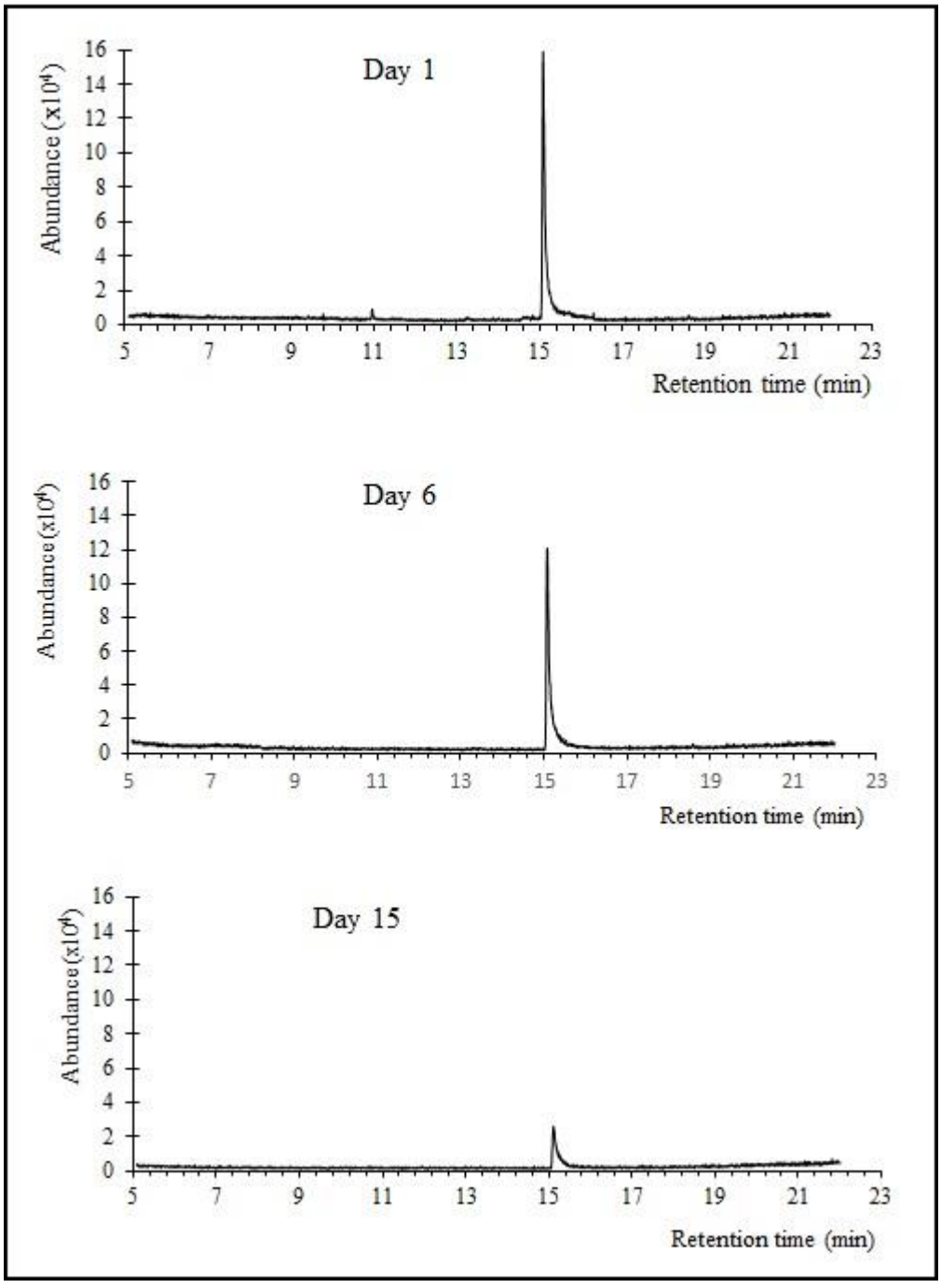


Figure 4-29. Representative chromatograms showing phenanthrene degradation profile

4.4.2 Pyrene degradation

To understand the performance of a single pure isolate compared with the performance of the whole enrichment (growth) culture, degradation of pyrene was carried out using pure isolate BC1 as inoculum in one degradation experiment, and using a small volume of its enrichment culture as an inoculum in another degradation experiment. In other words, in the isolation of pyrene-degrading isolate, there were five consecutive enrichment cultures after the initial incubation of one gram of soil in BH culture having pyrene as sole carbon and energy source. From the final (fifth) enrichment culture (EC), isolation on BH agar plate containing a layer of pyrene solution as carbon source yielded isolate BC1. One ml of the fifth EC was tested against 1 ml of concentrated solution of pure isolate BC1 in the degradation of 100 mg/l of pyrene. This was to be sure whether to use a pure single isolate or raw enrichment culture (having more than one isolate) in degradation of pyrene in the remaining experiments.

As shown in Figure 4-30, the two inocula took 40 days to achieve 80% degradation of four-ring pyrene. Pure isolate BC1 performed better (87%) with almost linear degradation rate which later slowed down as day 40 is approached. While EC may eventually catch up with BC1, it is not economical to wait that long and to manage the many strains that it would contain. These results also show that use of pure isolate is logical and, that pyrene degradation is far slower and more difficult than that of 3-ring PAH (phenanthrene) wherein as much as 95% degradation was achieved in only 15 days. Figure 4-31 shows sequential loss of pyrene concentration in the cultures as indicated by representative chromatograms from GCMS analyses.

The capacity of the isolates LA, LB and BC1 to degrade pyrene was assessed in 100 ml BH cultures containing 100 mg/L pyrene as the only carbon and energy source. It was observed that all the three strains degrade pyrene to between 50 to 62% (Figure 4-32). Isolate BC1 performed better, reaching about 62% degradation in 16 days. However, the observed difference may be as a result of higher initial population of isolate BC1 in inoculum solution. In fact, considering the initial inocula concentration/population, isolate LB grew faster reaching peak of exponential growth at day 10 (Figure 4-33). Growth in isolate LA is the slowest but keeps growing till the end of the experiment.

Bacterial growths in the culture was also estimated by measuring optical density (OD) of the culture solution at 600 nm wavelength. Figure 4-34 shows that there is an inverse relationship between bacterial population based on colony counts and growth estimation based on OD measurements. As bacterial population increases, the culture solution becomes clearer (resulting in lower OD values). Because pyrene is water-insoluble PAH, bacteria utilizing it as carbon and energy source will colonize the particles by adhering to its surface. When the particles are completely degraded or transformed to another soluble compounds, the cultures turbidity (and OD values) may increase. This implies that, counting of colony-forming unit is the reliable approach for assessing bacterial growth in water-insoluble PAH-degrading cultures.

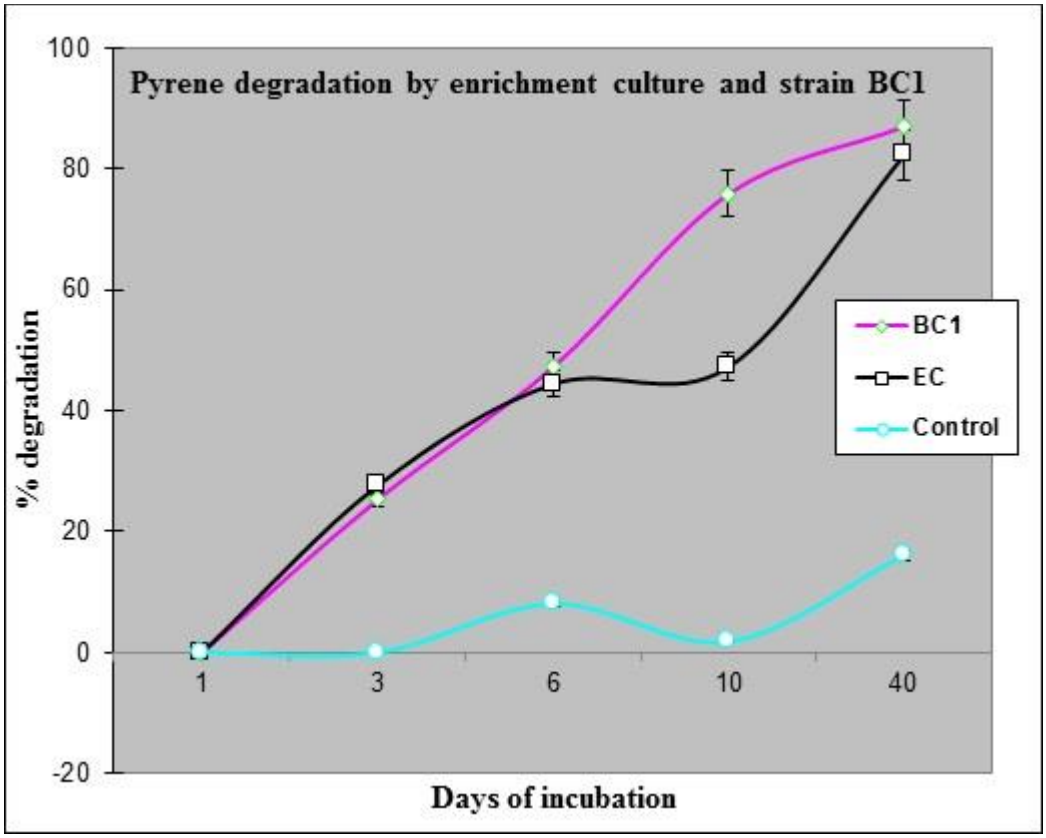


Figure 4-30. Comparison of pyrene degradation by enrichment culture (EC) and strain BC1 isolated from the EC culture

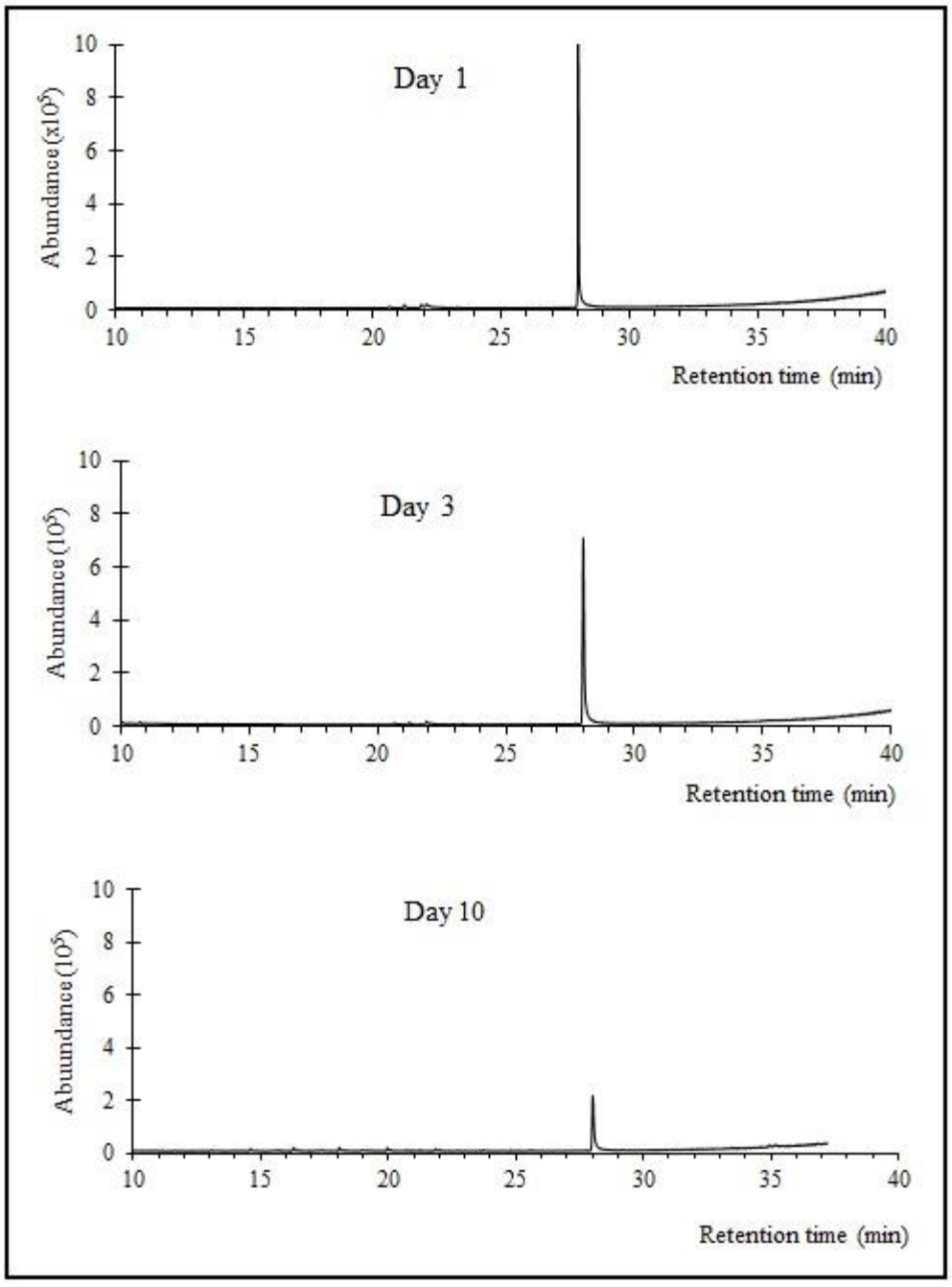


Figure 4-31.Representative chromatograms showing pyrene degradation profile by BC1.

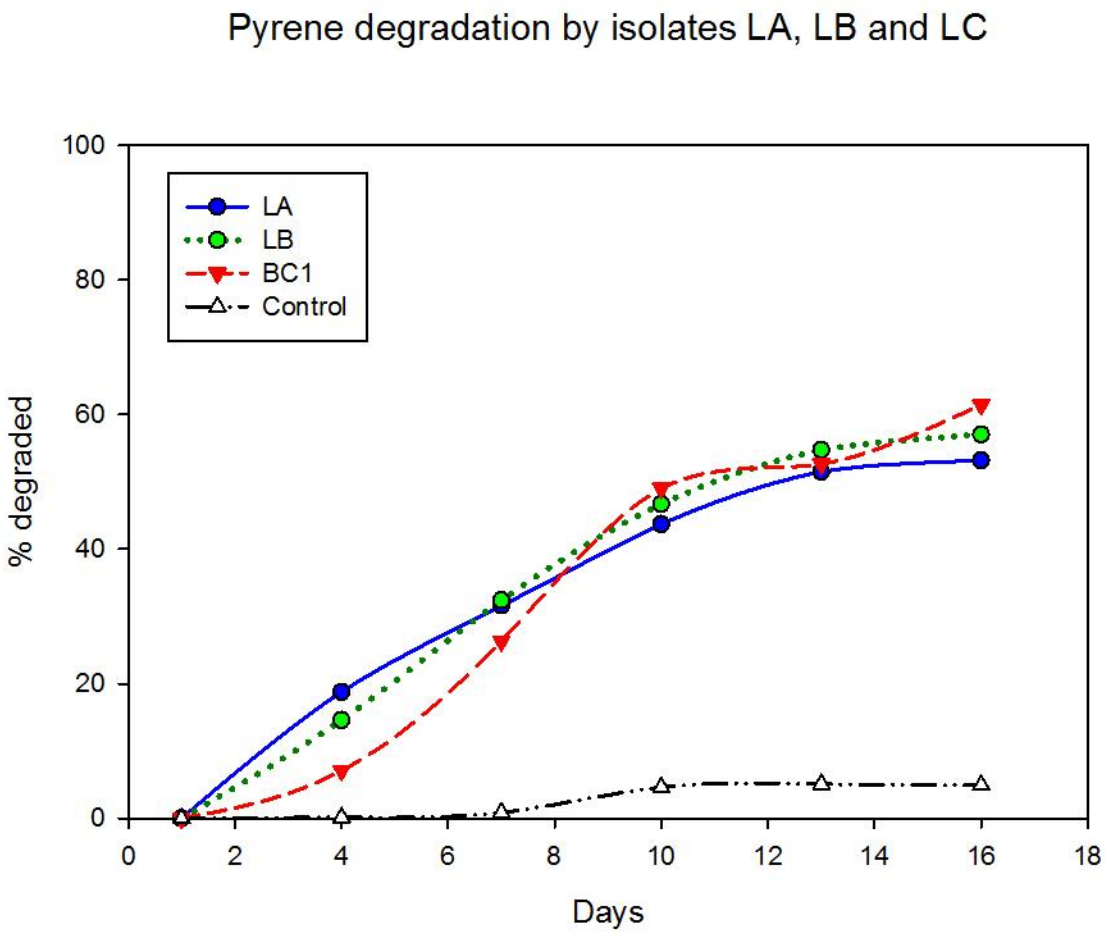


Figure 4-32. Pyrene degradation by isolates LA, LB and BC1

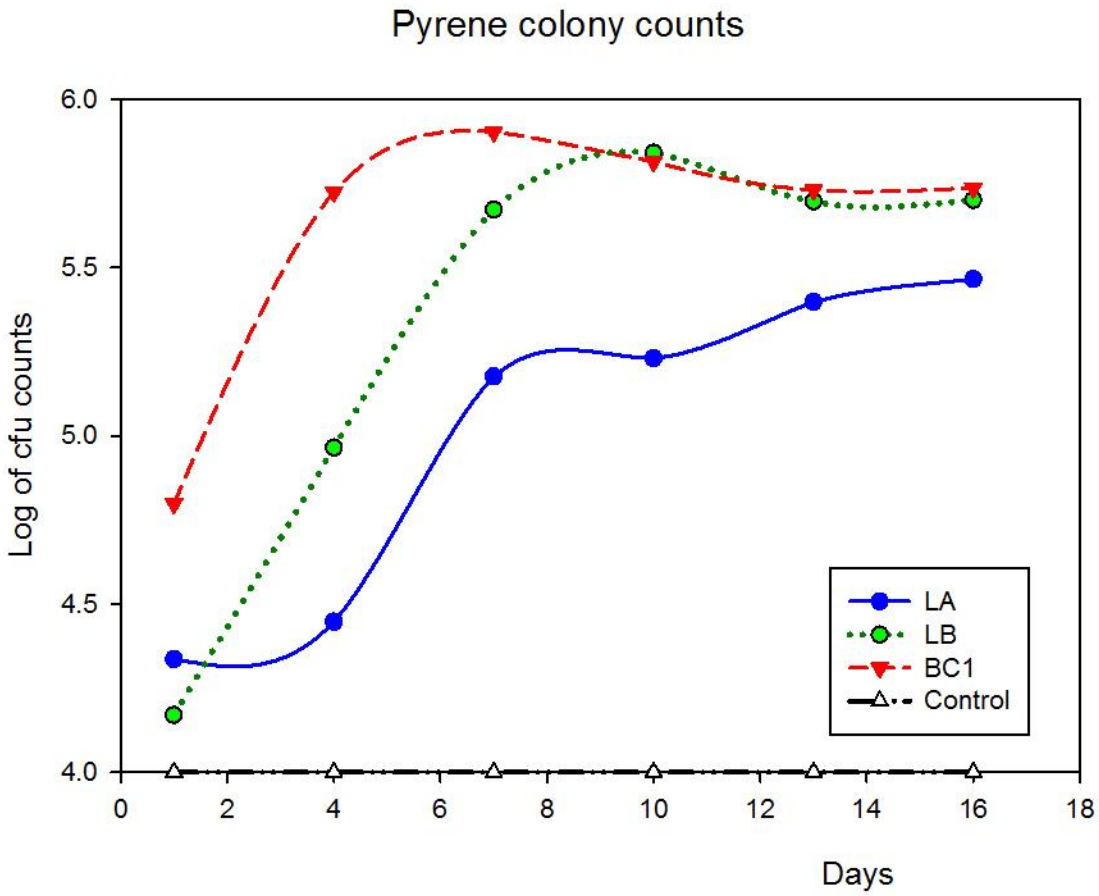


Figure 4-33. Bacterial growth curves of isolates LA, LB and BC1, based on population using log of number of colony-forming units per ml (cfu/ml).

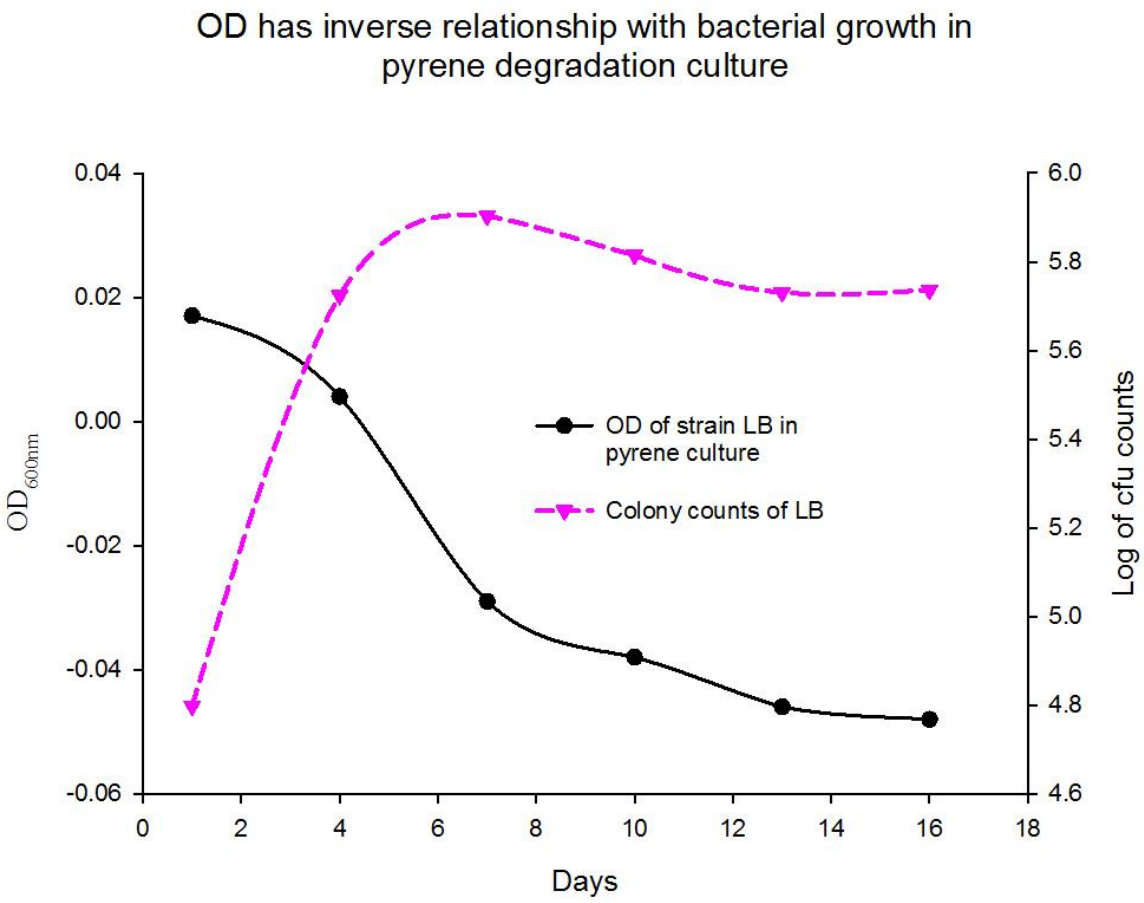


Figure 4-34. Relationship between optical density (OD) and logarithm of number of colony-forming unit per ml as a measure of bacterial growth. OD is not a good measure of growth in pyrene.

4.4.3 Diesel degradation

In other to evaluate the degradation of diesel by the isolates, two separate sets of experiments were carried out. The first set involved the use of unique isolates LA, LB and BC1 as inocula and then compare their performance. The other experimental sets attempt to understand how the components of diesel are individually degraded by strain specifically isolated for diesel degradation (i.e. its isolation from the original sediment sample used diesel as sole carbon and energy source).

4.4.3.1 Diesel degradation by isolates LA, LB and BC1

Three batches of BH culture solution containing 0.5% diesel as only carbon source were degraded separately using isolates LA, LB and BC1 as single inoculum in each batch. All of the three isolates grew in diesel-containing cultures (Figure 4-36) and degraded diesel in similar pattern (Figure 4-35). Isolate LA could however only degraded about 50% of the initial diesel concentration while isolates LB and BC1 degraded equal initial concentration to about 60% in 16 days. Loss in abiotic control is less than 15%, implying that the observed degradation is attributable to the bacteria utilizing the oil.

In the assessment of bacterial growths in diesel-containing cultures, either of colony-forming unit (cfu) counts or measurement of optical density (OD) is acceptable (Figure 4-37). However, it is advisable to take caution when using OD as remains of dead bacteria will still contribute to the culture's turbidity (measured as OD) especially after exponential stage of growth is exceeded (Figure 4-37).

There was a sharp but gradual pH changes in culture solution containing isolate LB. The solution pH starting from pH 7 at the start of the experiment, become acidic reaching the lowest pH of about 5 by day seven (Figure 4-38). The acidity is likely toxic to the strain reducing its population but it does not affect its degradation efficiency. The strain acclimatized quickly and stabilized its optimum population by day 10 while the solution pH is still below 6.0.

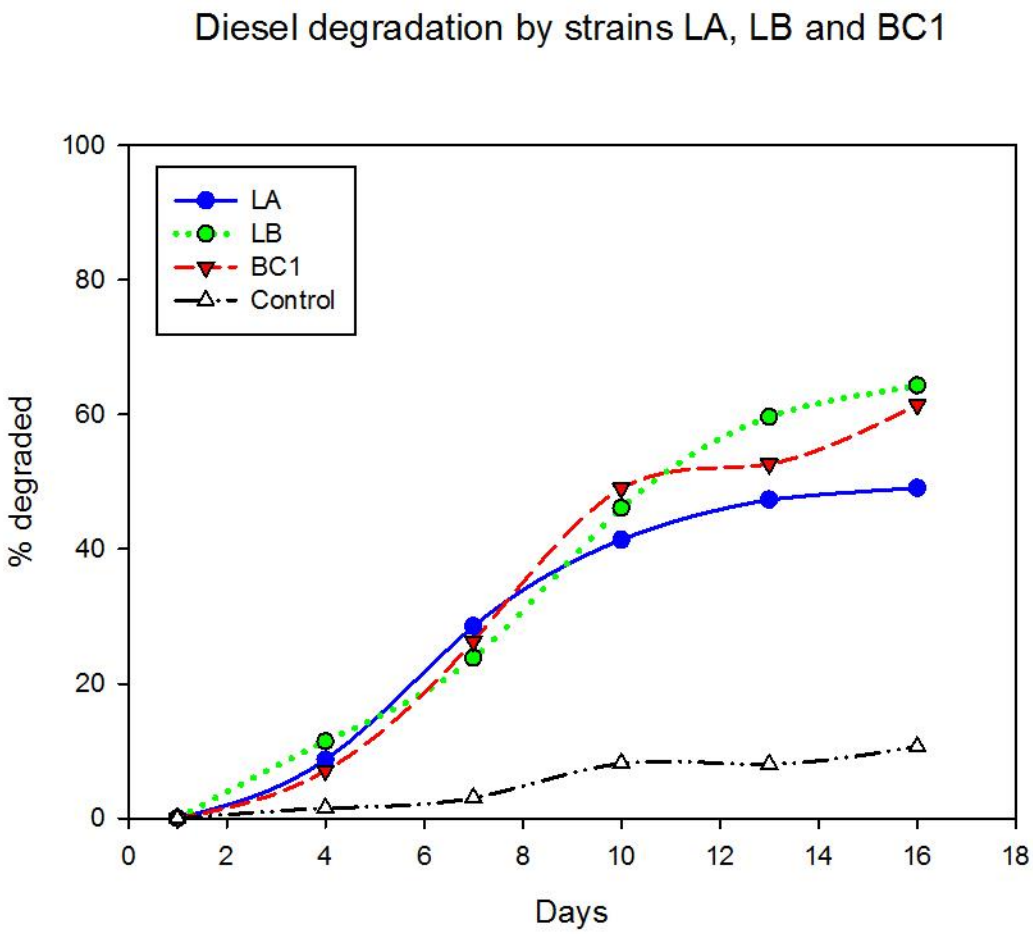


Figure 4-35. Comparison of diesel degradation by strains LA, LB and BC1

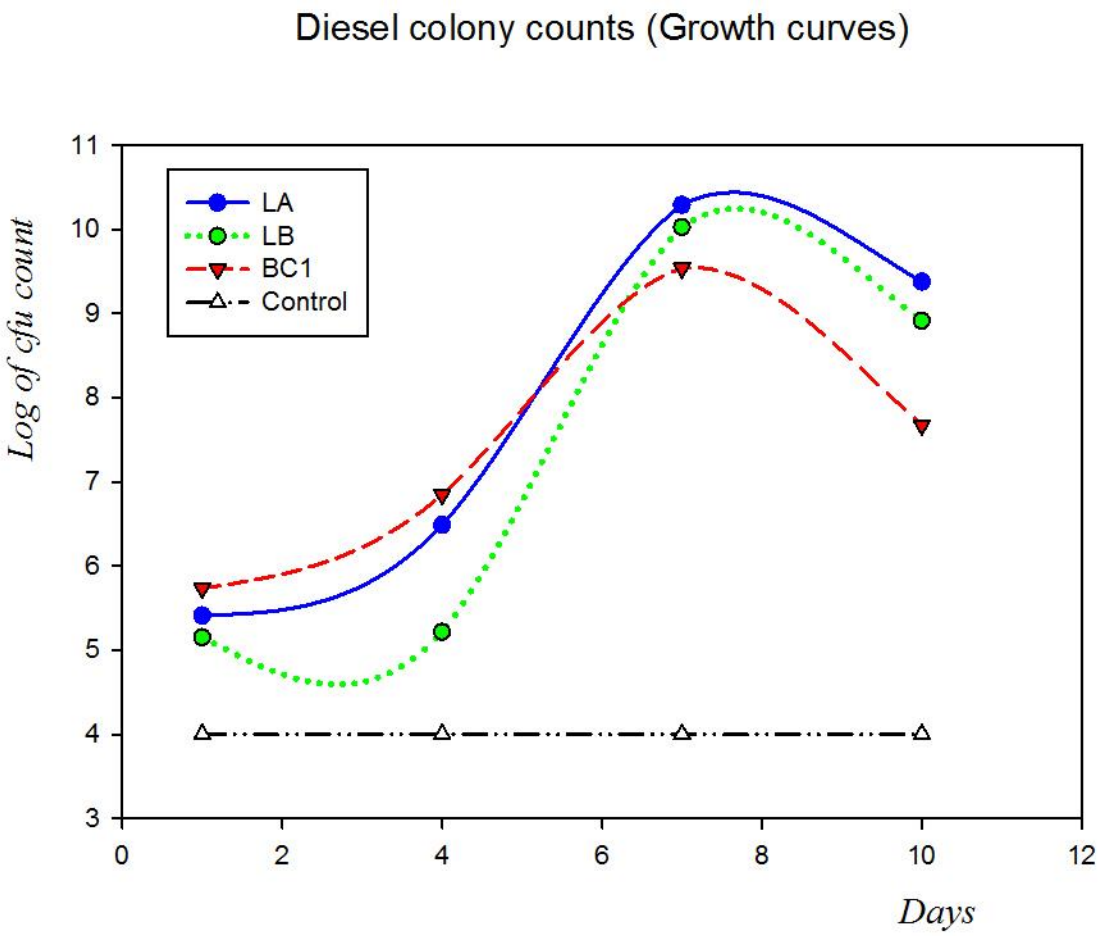


Figure 4-36. Growth curves of the isolates LA, LB and BC1 in diesel culture. Growth is estimated by bacterial population according to colony counts

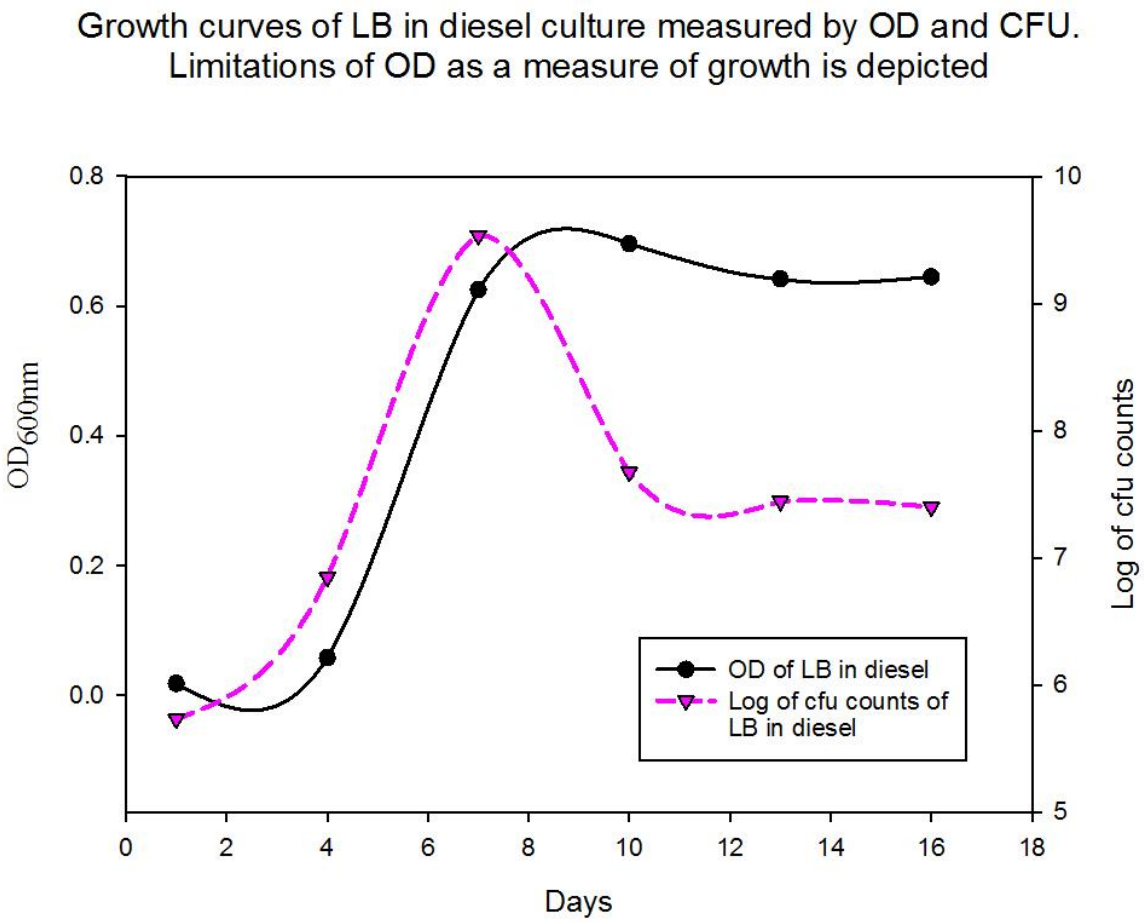


Figure 4-37. Comparison of growth curves in diesel culture by measuring optical density (OD) and by counting number of colony -forming unit

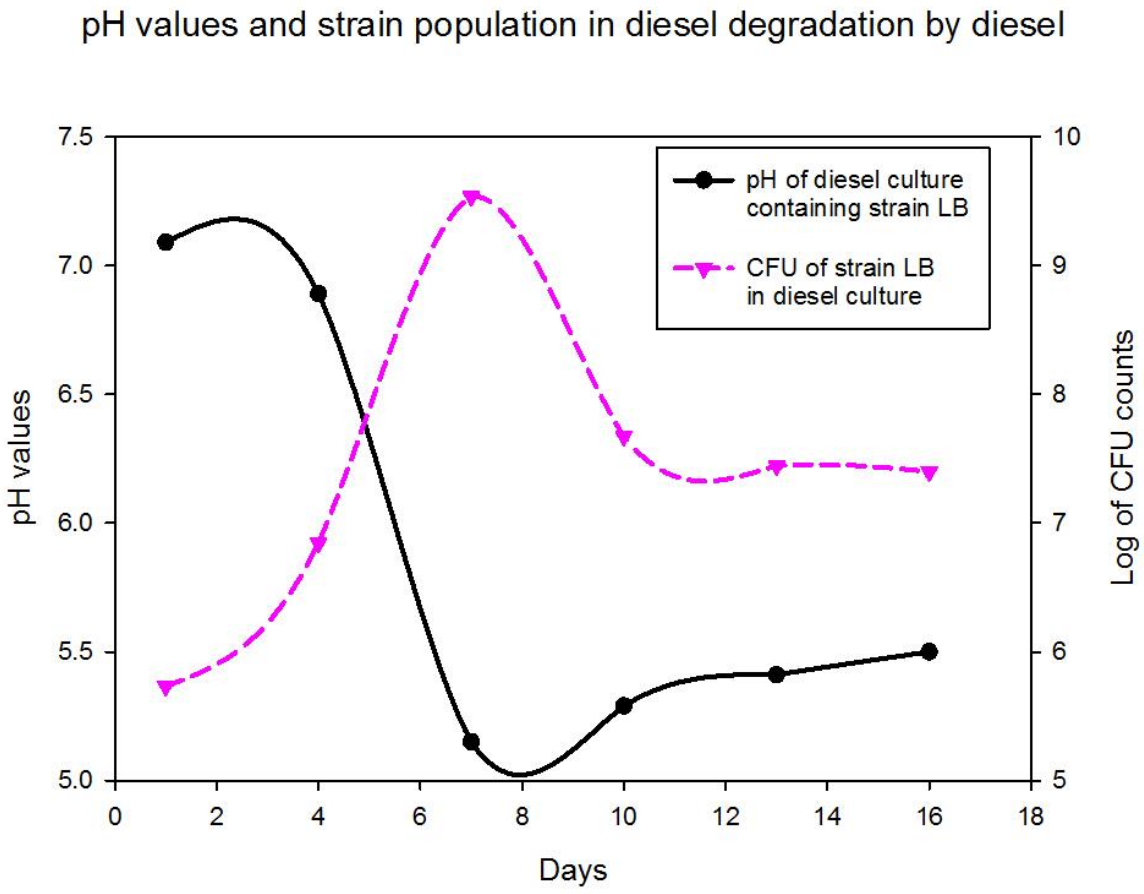


Figure 4-38. Variation of culture solution pH with bacterial isolate LB population

4.4.3.2 Degradation of diesel components by isolated *Pseudomonas aeruginosa* strains

Five isolates (strains of *P. aeruginosa*) were grown on diesel-containing degradation cultures to compare their growth rates on diesel, with a view to select best diesel-degrader. All the 5 strains, (the three phenanthrene-degrading isolates BC5, 6 and 7, and two isolates from diesel enrichments, i.e isolates DSA and DSB), show evidence of growth in the culture (Figure 4-39). Growth rate of isolate DSA however follows a typical bacterial growth rate reaching peak at 84 hours of incubation (Figure 4-39). Isolate DSB also grow fairly well, according to the culture's optical density (OD) measurements. All the three phenanthrene degraders did not improve their culture turbidity beyond a small sharp change that they caused after the first 12 hours. Isolate BC7 seemed to be the weakest to survive the pressure imposed by diesel oil. Optical density of the abiotic control culture remain relatively unchanged throughout the period of growth assessment.

Isolate DSA was subsequently therefore selected and used for degradation of diesel components. Its growth rate based on colony population (Figure 4-40) shows a very good correlation with the same assessment based on optical density (Figure 4-41). Bacterial population also reveal growth peak was reached after 84 hours similar to what was observed when OD was used. The curves follow a typical organism growth curve starting with about 12 hours of initial slow growing (lag) stage, followed by long hours of active growing and reproduction (exponential) phase and culminating in senescent phase at which population is kept at equilibrium.

In the actual diesel degradation assays, it was observed that about 90% of the diesel was removed by isolate DSA within 30 days (Figure 4-42). Some losses (around 40%) were also recorded in the abiotic control. Evaporation may contribute to the observed losses in the control. Assessing the losses according to the diesel component's number of carbon atoms, it was revealed that greatest losses were recorded in the components having fewer carbon atoms (Figure 4-43). For instance, only 10% of $C_{18} - C_{21}$ compounds was lost in abiotic control within 30 days while biodegradation achieved as much as 85% of the same compound within the same period. About 60% losses was noted in aromatic compounds. Compared to observed losses in abiotic control (>45%), the aromatic components degradation by the isolate is not really significant (Figure 4-43).

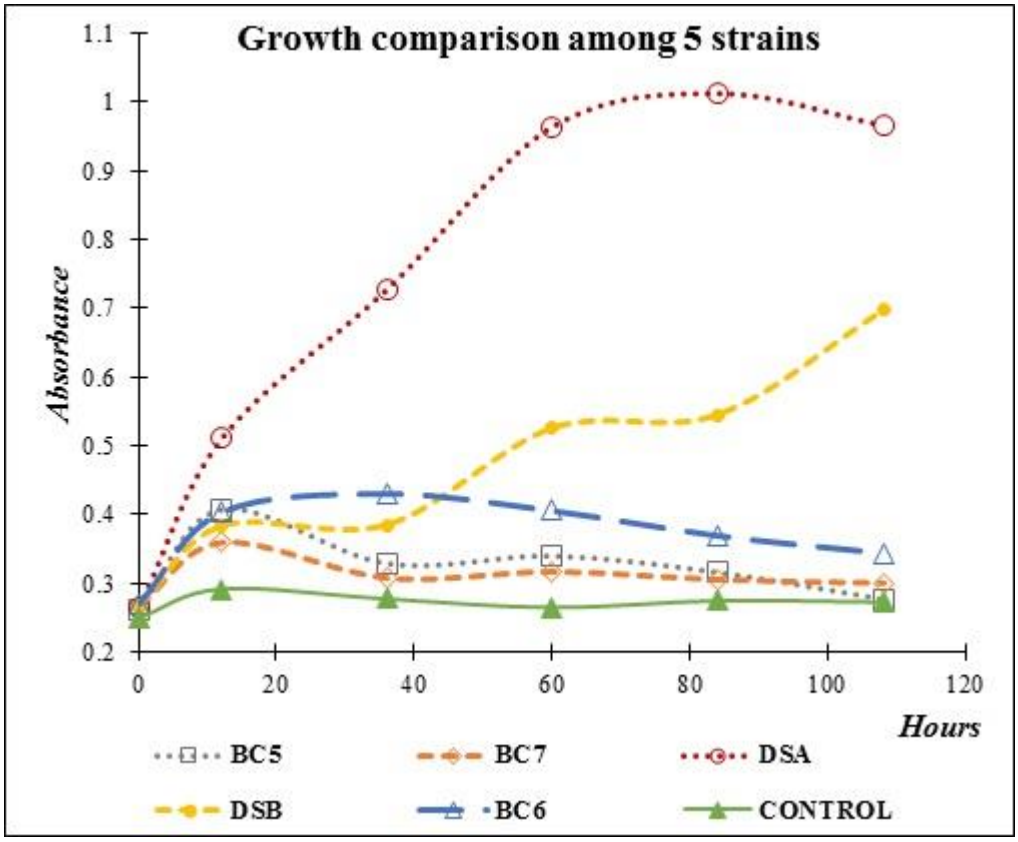


Figure 4-39. Growth curves of PAH-degrading isolates and diesel-degrading isolates as they grow in cultures containing diesel as sole carbon source.

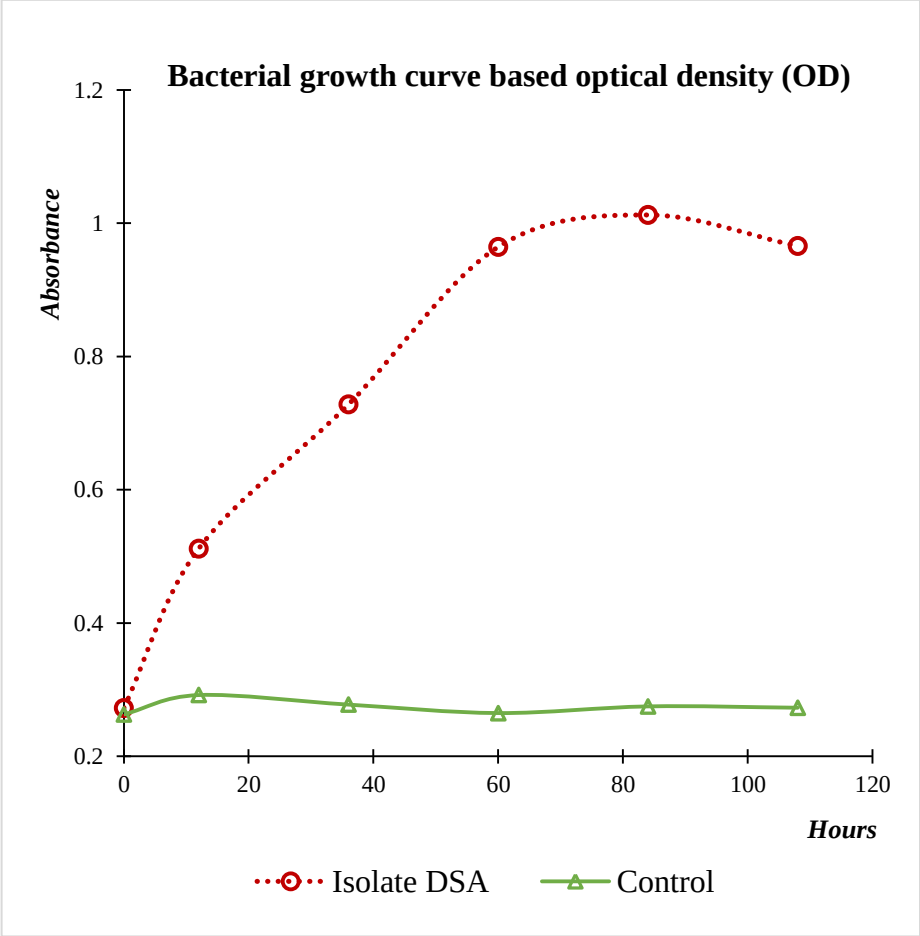


Figure 4-40. Growth curve of diesel-degrading isolate DSA in diesel-containing culture, based on optical density (OD) of the culture

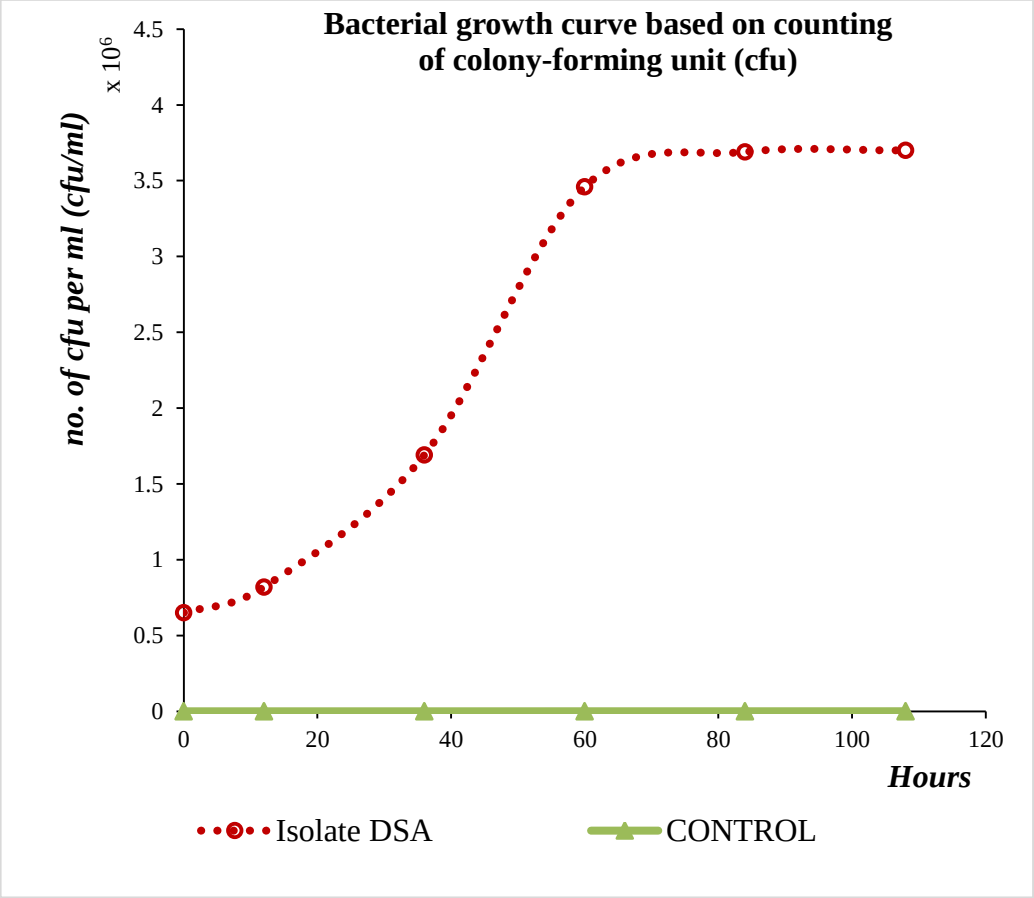


Figure 4-41. Growth curve for diesel-degrading isolate DSA in diesel-containing culture according to counting of colony-forming units

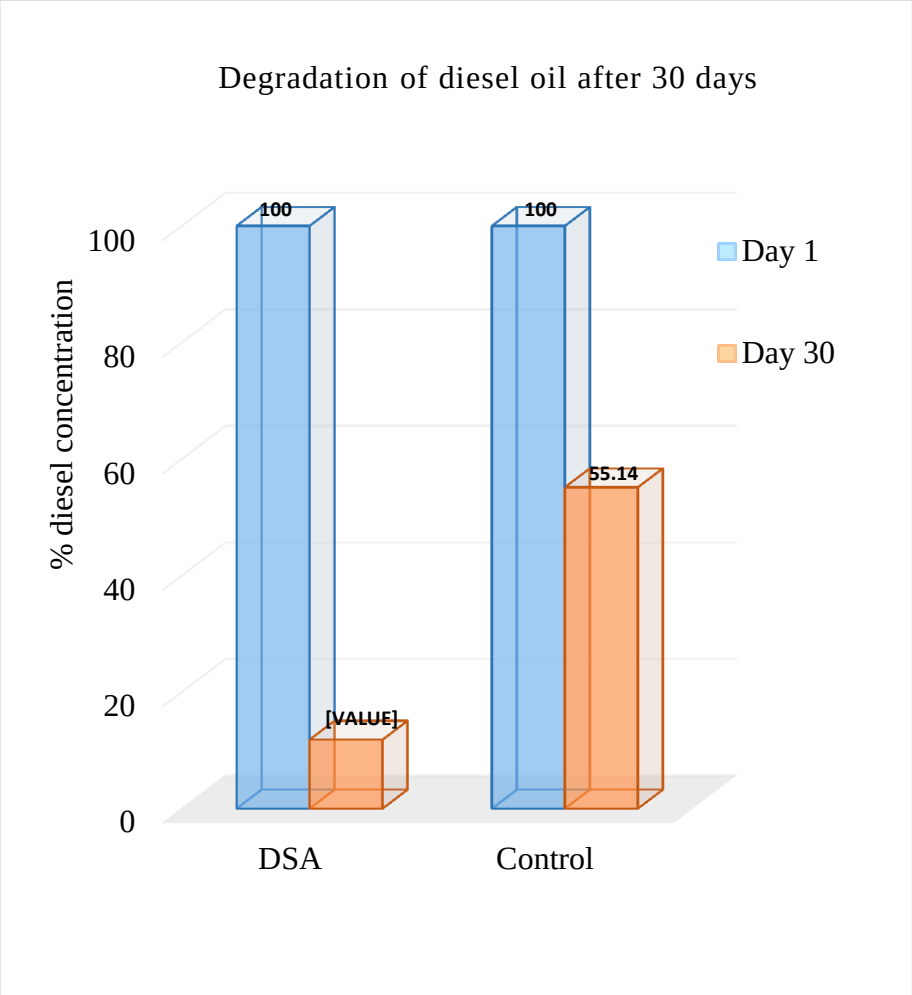


Figure 4-42. Degradation of diesel oil by isolate DSA after 30 days of incubation

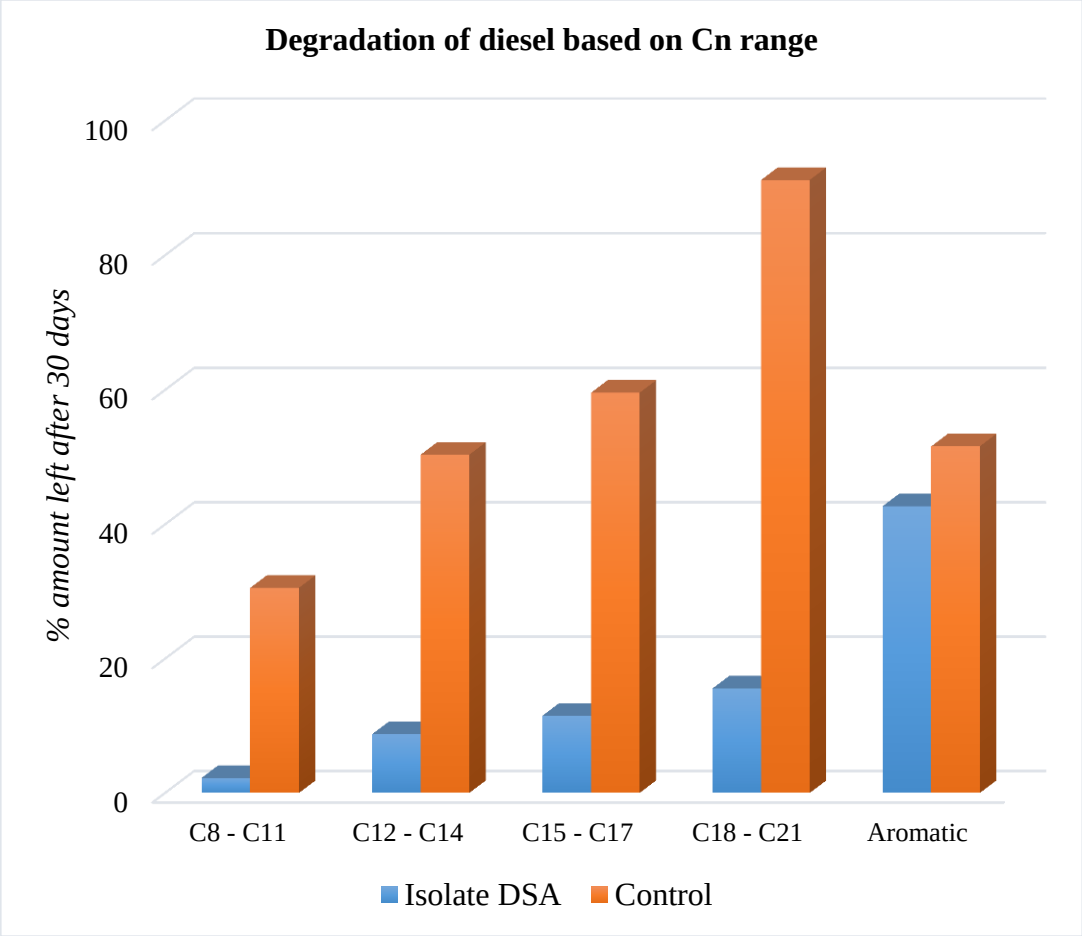


Figure 4-43. Degradation of diesel components according C_n range

CHAPTER 5

DISCUSSIONS

The uniqueness of Arabian Gulf in terms of its shallowness, pollution, physical and chemical properties has been widely studied and reported (Hassan et al., 1995; Price and Robinson, 1993; Price et al., 1994; Sheppard et al., 2010; Taher et al., 2012). These environmental factors, in addition to the extensive oil pollution caused by 1991 Gulf War, contribute greatly to the alteration of the Gulf's biodiversity especially its microbial communities (Al-Thukair et al., 2007; Barth, 2003). Despite the massive oil spill from the war, most of the coastal areas are left to self-remediation (Al-Thukair et al., 2007). Instinctively, the Gulf coast is still polluted and the microorganisms growing there are adaptable to crude oil toxicity and to varying but extreme physicochemical properties of the areas.

In this study, the Jubail coast where our samples were taken is still visibly polluted. Our measured PAH concentration indicated that only about 50% of the amount of aromatic components of the spilled oil reported shortly after the event (Fowler, 1993) is removed by natural attenuation. Of course, the recalcitrance of PAH implies that only a concerted remediation regime can remove it effectively. Other sediment physical and chemical parameters do not change much. The results recorded for our pH and other ionic concentrations were within the range reported in previous studies (Hassan et al., 1995; Taher et al., 2012) in a nearby location. From these studies and ours, we can therefore

conclude that any change in microbial diversity observed in the Gulf coasts is attributable mainly to the influence of oil pollution.

Isolation from enrichment cultures commonly select only strains that are able to adapt to pressure imposed by high concentration of toxic hydrocarbon used as carbon and energy source. In other words, bacterial species/strains that cannot withstand the pressure will miss isolation. The diversity and proportion of oil-degrading bacteria in a microbial community oil-polluted sites increase with prolonged exposure to oil or its product (Nair et al., 2008). In our study, no bacteria was isolated from sample location A when pyrene was used as sole carbon source. Location A is a beach and not as polluted as other two sampling locations. Hence, bacteria that can utilize higher molecular weight (HMW) PAH like pyrene as sole energy source is absent in the microbial community of the beach.

The prevalence of rod-shaped bacteria in biodegradation cultures is very common in literature (Chaudhary et al., 2011; Ferradji et al., 2014; Lavania et al., 2012; Nair et al., 2008; Obayori et al., 2009). In many of the studies, the rod-shaped strains are biochemically gram negative (Lavania et al., 2012; Nair et al., 2008; Obayori et al., 2009). In fact, *Streptomyces sp.* and *Bacillus sp.* are only a few of the rod but gram positive bacteria that are still good degraders (Al-Sharidah et al., 2000; Chaudhary et al., 2011; Ferradji et al., 2014; Reunamo et al., 2013). All the 15 isolated strains in our study are rod-shape, gram negative bacteria. The dominance of gram negative rod isolates can be explained by its shape and enrichment procedure. During enrichment and sub-culturing, only strains with prominent morphologies are selected and further purified. This process favors bigger and fast-growing strains that are often rod in shape. Cocci are too small and

may miss the chance of being selected for further sub-culturing. The lucky sphere-shaped strains may be good degraders but they might take more time to establish sufficient population for degradation in a culture.

Sequencing of the conserved region of organism DNA is one of the most reliable method of identifying organisms. In bacteria, 16S rRNA is the conserved region and the commonly sequenced genes for identification. Molecular analysis of the isolates in this study indicated that all the isolates are in the phylum *Proteobacteria*. Isolation by repeated enrichments and serial culturing have previously been reported to specifically select or favor Proteobacteria, wiping out completely initially dominant members of Firmicutes especially *Bacillus sp.* (Mao et al., 2012). However, the presence of members of three classes of this phylum implies diversity of microbial communities in our oil-polluted sampling location. Furthermore, we noted a dominance of genus *Pseudomonas* among the isolates. Previous reports have also shown dominance of *Pseudomonas sp.* especially *P. aeruginosa* in studies involving isolation of degraders from oil-polluted locations (Abed et al., 2014; Chaerun et al., 2004; Garcia-Valdes et al., 1988; Ghosh et al., 2014; Jacques et al., 2008; Luo et al., 2009; Niepceron et al., 2010; Obayori et al., 2009; Whyte et al., 1997).

P. citronellolis is another species of *Pseudomonas* isolated from this study. This species is seldom present among isolates from oil-polluted areas. However, it has been found as the predominant isolates from oily-contaminated sites (Bhattacharya et al., 2003) and as one of the important but rare polymer degraders (Bode et al., 2001). Even though, ordinarily, isolation of *P. citronellolis* strains is not common from enrichment cultures having

aromatic hydrocarbon as carbon source. Nevertheless, since isolation was carried out after repeated 7 days of culturing, the species might establish itself after the aromatic ring of PAH is opened up by other degraders. This explains the presence of *P. citronellolis* as one isolate from 'aromatic' compounds that it was not expected to utilize.

Isolates BC1 and LA are identical to *Ochrobactrum intermedium* and *Cupriavidus taiwanensis* respectively. While *O. intermedium* is commonly found in hydrocarbon degradation studies as culture-independent strains (Festa et al., 2013), *C. taiwanensis* is not commonly reported in PAH biodegradation. It has however been in utilized in the phenol degradation wherein it tolerated up to 900 ppm of it (Chen et al., 2008). Similarly, in the KEGG pathway model (Kanehisa and Goto, 2000), *C. taiwanensis* are believed to have various degradation pathways for PAHs and most aromatic compounds except bisphenol, ethylbenzene and furfural (2-Furaldehyde) (Zuleta et al., 2014).

According to phylogenetic relationships of the isolates, the 12 isolates are closely related to *P. aeruginosa* but they behaved differently in different hydrocarbons showing that they are different strains of the same species. For instance, the two isolates from diesel (DSA and DSB) grew very well on diesel, their pattern of growths differ. Similarly, the three highly ranked isolates BC5, BC6 and BC7 were also identified as *P. aeruginosa* but have different colony morphologies and phenanthrene degradation rates. By these, we conclude that they are distinctly different strains of the same species, *P. aeruginosa*. The phylogenetic ancestors and relatives of isolate BC1 are mainly pathogenic species, in the *Brucella sp* group that are infectious to animals and human (He, 2012). Many strains in *Ochrobactrum* species are known to be harmless and our isolate BC1 is most closely

associated with *O. intermedium* with no known pathogenesis. Similarly, isolate LA (close relative of *Cupriavidus taiwanensis*) has *Bulkhoderia sp.* and *Ralstonia sp.* as one of the ancestors and relatives respectively. Within the family *Bulkhoderiaceae*, there are hydrocarbon-degrading members as well as N-fixing bacteria inhabiting root noodles (Amadou et al., 2008; Vandamme and Coenye, 2004; Zuleta et al., 2014).

Degradation efficiencies vary within our isolates, and for different hydrocarbons used as carbon source. Among our *P. aeruginosa isolates*, BC5, BC6 and BC7 were found to be good phenanthrene degraders but not so efficient in diesel degradation. While, another strain of *P. aeruginosa*, isolate DSA was an excellent diesel-degrader but with poor performance on aromatic compounds. The strains/isolates have developed adaptability to utilize optimally, their carbon source from which they were isolated. This was also the case with previously reported *P. aeruginosa* strains isolated with specific hydrocarbon as carbon source (Chaerun et al., 2004; Ghosh et al., 2014).

On the other hand, Isolate LB (*P. citronellolis*) isolated from culture having combination of 3 PAHs (anthracene, pyrene and phenanthrene) performed very well across board. It degrades over 90% phenantherene, about 60% pyrene and over 60% diesel within a period of 15 days. The isolate's performance can be attributed to the activity of a (surfactant-like) substances it produces. The substance caused a sharp reduction in pH of its diesel-containing cultures. Previous studies implicated *P. citronellolis* as a very good producer of surface tension reducing substances (Fall et al., 1979; Jacques et al., 2008; Santos et al., 2008). The chemical substances, enzymes, substantially contributes to making available the carbons in cyclic and acyclic hydrocarbons to metabolic use by the bacteria. This

feature suggests that the use of *P. citronellolis* in bioremediation reactor may serve as an alternative to the use of chemical surfactants.

Pyrene biodegradation is slower than phenanthrene because of the additional aromatic ring that confers addition stability and recalcitrance. Although all the tested strains degraded pyrene, BC1 (*Ochrobactrum intermedium*) and LB (*P. citronellolis*) performed better. Like *P. citronellolis*, the colony of *O. intermedium* was also observed to be whitish and produced to be producing a kind of surfactant. The substance was identified as a kind of bio-emulsifier called exopolysaccharide (Ramasamy et al., 2014). Deductively, bacterial ability to produce secretions enhances its potential for degradation of HMW PAHs.

From our study, we also found that bacterial growths and PAH degradation have a direct relationship. However, growth assessments must be done with caution. In PAH cultures, the best assessment of culture growth/population is by counting colony-forming unit (cfu). Use of culture density may be deceptive. While there is no definite relationship between culture density and bacterial growth in phenanthrene-containing cultures, there is a somewhat negative relationship between pyrene-containing cultures and density (Figure 4-34). This was explained as cell size augmentation caused by cell weight increase due to accumulation of HMW compounds into the cell cytoplasm (Madueño et al., 2011). This makes the bacterial cells heavy and tend to settle at the bottom making the liquid less turbid. Also, bacteria utilizing water-insoluble PAH do not stay in the solution, rather, they adhere to the surface of PAH particles. This further reduces the liquid turbidity, implying that OD is not suitable for larger size PAHs as a growth assessment method.

Nevertheless, OD can reliably be used for growth assessment in liquid or water-soluble hydrocarbons. Growth curves of all strains tested for diesel degradation follows a typical organism growth curves having; initial lag phase, an exponential growth phase and final slow (senescent) phase. Despite that both OD-based and cfu-based growth curves displayed the triphasic shapes (Figure 4-37), when population starts to fall at day 7, it appeared to be stable or constant on OD-based curve. The result shows that while cfu accounts for living bacterial population, OD takes care of both living and dead ones.

In this study, we also demonstrated how *P. aeruginosa* degrade different components of diesel. The isolate DSA utilized and degraded all components of diesel but it is a poor degrader of aromatic compounds. GCMS analyses of the aliphatic compounds revealed that components with 18 to 21 carbons ($C_{18} - C_{21}$) were the best utilized and degraded components. While most of the losses recorded in diesel components with few carbon atoms (i.e $C_8 - C_{14}$) were not attributable to bacteria activity. Previous studies suggest that the lighter components are more toxic to bacteria but fortunately they evaporate quickly (Eriksson et al., 1998; Mukherji et al., 2004; Ramasamy et al., 2014). From our study, the increasing order of degradation of aliphatic components of diesel is by *P. aeruginosa* strain DSA-01 is: Aromatic < $(C_8 - C_{11})$ < $(C_{12} - C_{14})$ < $(C_{15} - C_{17})$ < $(C_{18} - C_{21})$.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

CONCLUSIONS

In this study, hydrocarbon-degrading bacteria were isolated from Arabian Gulf coastal sediments polluted with oil during 1991 war. The isolates were characterized morphologically with microscopy as rod and gram-negative strains having varying colony morphologies. Molecular analyses by sequencing 16S rRNA revealed that majority of the PAH degrading strains in Arabian Gulf belong to genus *Pseudomonas*. Although the isolates are diverse, they have close evolutionary relationship or phylogeny with species that have been reportedly found in oil-polluted sites around the globe.

Two strains, BC5 (*P. aeruginosa*) and LB (*P. citronellolis*) were noted as excellent degraders of phenanthrene removing about 95% within 15 days. Pyrene degradation is much slower and BC1 (*Ochrobactrum intermedium*) was among those that biodegrade it very well. Despite that all tested isolates grew on, and utilized diesel, isolate DSA (*P.aeruginosa*) was ranked best, reducing most diesel components very efficiently except aromatic components.

Finally, this study established that population counts by colony forming unit (cfu) is more reliable than optical density (OD) assessment in estimating bacteria growths in PAH-degrading liquid cultures. Even though it may be acceptable for cultures containing soluble (or liquid) hydrocarbons, cfu method is better and thus recommended.

RECOMMENDATIONS

The bacterial isolates should be tested on model polluted soils and then scaled up for use in remediation bioreactor (*in situ*) or for application on the field.

We recommend optimization of biodegradation conditions for the best-performing isolates while being tested on model polluted soil rather than in liquid cultures. Different combination of the strains, as consortium, should be assessed in the optimization.

The strains are good candidates of commercial oil-degrading microorganisms (ODMs). Attempts should be made to partner with relevant firms with a view to commercializing the excellent degraders as indigenous ODMs.

Finally, we recommend further research to study the metabolites and pathways of the isolates' degradation, and identification of the specific gene(s) that are responsible for the degradation. This will enable future cloning of the isolates to have abundance of specific genes for specific hydrocarbon biodegradation.

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APPENDICES

APPENDIX A: 16S rDNA SEQUENCES

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APPENDIX B: CALIBRATION CURVES

Calibration curve of instrument (SiteLAB) used for soil EPAH measurements

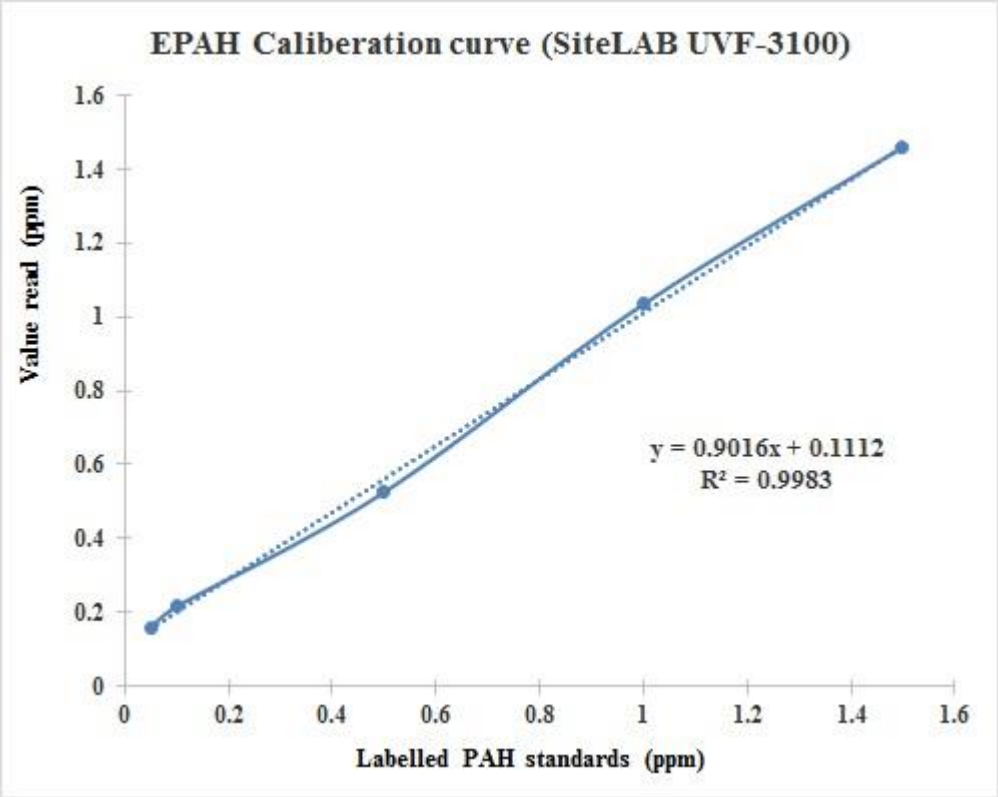

Figure B-1. EPAH calibration curve on SiteLAB UVF-3100

Phenanthrene calibration curve on GCMS

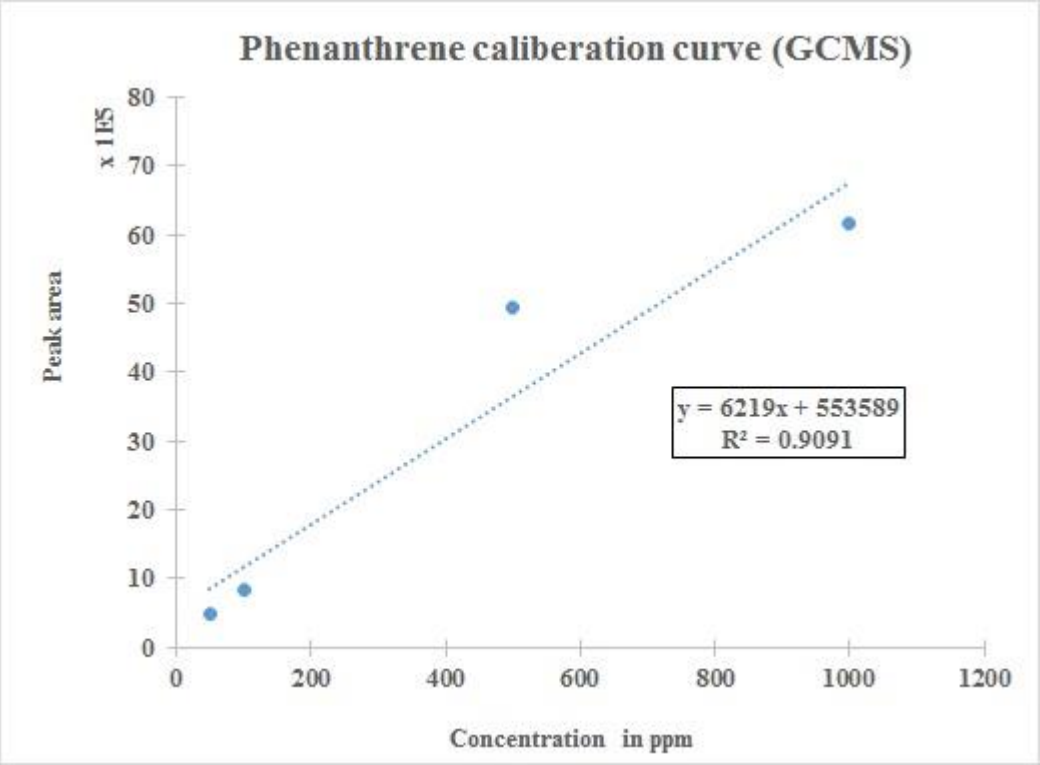

Figure B-2. Phenanthrene calibration curve on GCMS

Pyrene calibration curve on GCMS

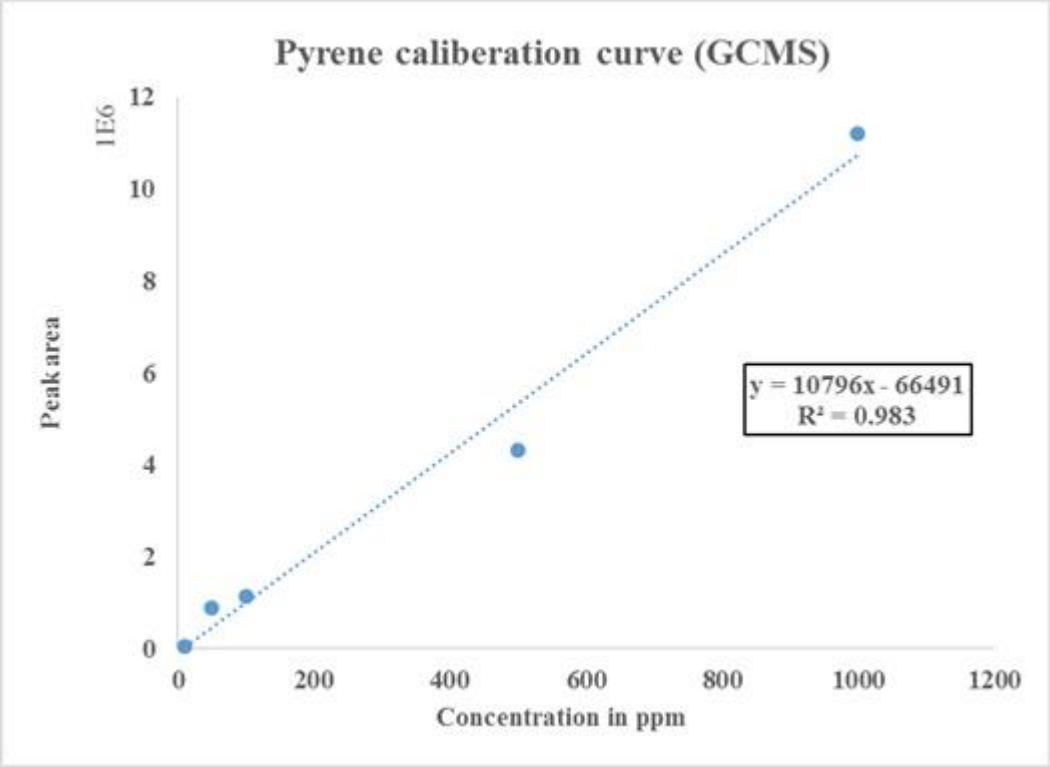

Figure B-3. Pyrene calibration curve on GCMS

Vitae

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